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BRITISH MEDICAL BULLETIN

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RECENT RESEARCH ON VITAMINS

Scientific Editor . . . . . B. S. PLATT

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NEURO-OTOLOGY

Scientific Editor . . . . . C. S. HALLPIKE

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PHYSIOLOGY OF VOLUNTARY MUSCLE

Scientific Editor . . . . . W. D. M. PATON

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BRITISH MEDICAL BULLETIN
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Scientific Editor: *B. S. Platt*

Introduction

This volume of essays was planned by a committee of which Sir Edward Mellanby acted as Chairman. It cannot have happened often that a man plans a volume of this nature which then becomes his epitaph, and he himself was intending to make a personal contribution in addition to writing this Introduction. In many ways it is singularly appropriate that this work should have formed one of his last activities, because it has so much that is characteristic of the man. There is for instance the emphasis upon a forward outlook which does not leave touch with the past, and the encouragement of the younger folk to find new things. Always Mellanby emphasized the fact that it was blind to emphasize the increase in weapons of destruction without stressing the enormous improvement of medical science, as the results of research in recent years. As an illustration, in the nutritional field, which Mellanby made so much his own, it is hard to say how many young people owe their better teeth at the present day to the results of Mellanby's definition of the antirachitic vitamin, to Lady Mellanby's work, and to his early and later work on calcium metabolism. We are apt to forget the debt which we owe to studies of this nature. They have been here admirably reviewed and brought up to the present time by R. A. McCance who has himself contributed so much.

The Appreciation of Mellanby by Sir Charles Harington in this volume makes unnecessary any comprehensive allusion here to his life and work. His influence in connexion with the Accessory Food Factors Committee of the Medical Research Council and the Lister Institute of Preventive Medicine is admirably reviewed by Harriette Chick and M. Hume, who have put the whole work of this body in perspective. None is better fitted for this than Dame Harriette, who for years was the mainstay as Secretary of this Committee, of which Sir Edward was for long the Chairman; with his enthusiasms he was always a critical Chairman, and when I succeeded him as Chairman I always valued his comments. I can remember many occasions where such critical comments stabilized some situation along the right lines, as they must so often have done in other fields while he was Secretary of the Medical Research Council. As Chairman of the Accessory Food Factors Committee naturally he kept up a continuous and active interest in the whole field of vitamin research, an interest which we see reflected in the various papers comprising this number of the Bulletin.

The subject of vitamins has undergone much change since the early days when Hopkins, McCollum, Mellanby and McCarrison worked on nutrition. As Dame Harriette says, " the newer knowledge of nutrition " is no longer new; nor can it be said that it forms a subject for a lecture to revive the student when interest may be flagging. Vitamins have grown up; we are not now concerned with the misty atmosphere of whether they exist, and what entities they may be; nor even are we so much bothered about how many there are. We know now that there is a gradation in the amounts of various substances required in the daily diet, and amongst these substances vitamins are those factors required in very small amounts. We also know that many of these form part of definite coenzymes. A majority of them have probably now been discovered; still it would be a bold person who would say how close we were to the ends of discovery of new food factors. Nevertheless the emphasis is shifting to the more accurate study of the physiology and the pathology of their action; it is inevitable that the complexities of the interaction of nutritional factors will occupy the minds of scientists for a long time.

The subject of vitamin A metaplasia had long interested Mellanby and had formed the subject of his Croonian Lecture in 1943; lately this work received a fascinating turn with his new research upon tissue cultures with Dr Honor Fell, who here discusses this work in an article on the effects of vitamin A in modifying the growth of tissue cultures. It is indeed tragic that Mellanby should not be with us now to continue to participate in this important work. From this angle it is appropriate that L. J. Witts should discuss effects upon the blood and gastrointestinal tract, that C. H. Best and colleagues should discuss effects upon the liver, and A. Pirie those upon the eye. All this is in line with an emphasis upon the importance of studying vitamins in their relation to physiology and pathology as well as to their more purely chemical aspects.

Intimately related to the work of the Accessory Food Factors Committee is also the article upon the preservation of food by L. W. Mapson. Here we get in perspective a plan for the maintenance of vitamins during the processing of food and its storage. With all his interest in the subject of accessory food factors, Mellanby did not allow himself to get out of perspective. I well remember his saying towards the end of the Second World War that he thought the time had come to write an article on the " unimportance " of vitamins; so impressed had he become with the minute amounts of vitamins that often appeared to function, the long periods of storage of fat-soluble vitamins as seen in the Medical Research Council experiment on volunteers deprived of vitamin A, and the small amounts of vitamin C required to protect against scurvy as seen in another such trial, in Sheffield.

A research number like this would have been incomplete without some reference to individual vitamins. Much has been added to our knowledge of the chemistry of vitamins since B. C. P. Jansen and W. F. Donath in Java in 1925 first crystallized the vitamin now known as thiamine. Not one has reached the activity, weight for weight, of vitamin B_{12}; the small amounts in which vitamins are active has always been one of the romantic things about them, but in this respect vitamin B_{12} surpasses them all in the minuteness of the amounts which can be active. The story by E. Lester Smith of its isolation, and more recently of the determination of its structure by the combined effort of chemists and *x*-ray crystallographers (Sir Alexander Todd, Dorothy Crowfoot Hodgkin and co-workers) must assuredly form one of the more striking stories of scientific endeavour in our time. Then we have an account of the present position of vitamin C research by L. J. Harris, and of vitamin E by T. Moore. Of the former, in spite of all the work done, there is still much more to discover; of the latter vitamin we do not seem to have heard the last word, because there is a suggestion (still to be properly established) that it may play its part in averting some of the troubles of old age. Again we have an authoritative statement upon carotenoids and vitamin A by R. A. Morton & T. W. Goodwin, and one by A. Albert upon the effects of antivitamins in their relation to chemotherapy.

M. E. Coates, S. K. Kon & J. W. G. Porter discuss aspects of the problems of vitamins in animal nutrition from the standpoint of factors such as the thiamine-destroying factors which affect the availability of vitamins in the diet, and also the important question of the intestinal synthesis of vitamins; the influence of intestinal factors was recognized early, but recent research, especially in relation to vitamin B_{12}, has taught us how much more we must know about this both in the animal and in man if we are to understand a large chapter of nutrition.

H. M. Sinclair contributes two articles, upon skin and upon the nervous system respectively, bringing to our notice much modern work. In regard to the latter, Dr Sinclair has interestingly revived the controversial idea that the pyruvic acid which accumulates may be toxic and so give rise to nervous signs. This idea that vitamin deficiencies must involve toxic factors was much argued in the early days of vitamin research and was a worry to nutritional workers because clinicians could not conceive of disease as originating without a positive toxic agent. To use an analogy, the failure of the engine must be due to grit in the engine rather than to the loss of an essential part of the machine. When H. M. Sinclair and R. H. S. Thompson with myself discovered the connexion between thiamine deficiency and pyruvate metabolism, the possibility that the normal metabolite pyruvic acid might actually be the toxic agent had to be considered. Admittedly it is most difficult to decide between cause and effect in physiology. At present it seems to me that there are no sufficient grounds for believing that the signs of deficiency are due to a toxic agent; failure in the energy supply seems to be cause enough for initiating the dysfunction; this view is based upon general experimental results and especially upon experiments by G. D. Lu, a colleague of Platt's. Professor Platt himself has contributed an essay on vitamins in nutrition, in which he has given a much clearer perspective than can be given in this Introduction. Dr Sinclair has rightly stressed in the final paragraph of his first paper the prophetic nature of Mellanby's thoughts upon fruitful lines of research.

This number would have been more complete if it had contained references to the direct part played by vitamins as co-factors in biochemical (enzyme) systems; but this aspect has been reviewed recently in the Bulletin.[1] Furthermore, the work done on vitamins in relation to micro-organisms has been discussed thoroughly in earlier numbers of the Bulletin by M. Stephenson, E. F. Gale and D. D. Woods.[2]

In an Introduction of this nature it is not possible to do more than draw the reader's attention to this interesting cross-section of some of the work in progress in the field today; but any time taken in perusal should amply repay with an enlargement of the perspective of the reader and indicate how knowledge can be deepened.

Rudolph Peters

[1] Some Aspects of Enzyme Research (*Brit. med. Bull.* 1953, Vol. 9, No. 2).
[2] See New Currents in Biochemistry (*Brit. med. Bull.* 1948, Vol. 5, No. 4-5) and Some Aspects of Enzyme Research (*Brit. med. Bull.* 1953, Vol. 9, No. 2).

C. Mull & Co., Lucknow

SIR EDWARD MELLANBY
1884–1955

Edward Mellanby

IN happier circumstances Sir Edward Mellanby would himself have taken an even greater part in the production of this number of the *British Medical Bulletin*. Now that it has to be regarded more in the light of a memorial volume to him, it is fitting that we should recall the character and achievement of the man, but for whose work in the laboratory and the office much of any modern review of nutrition would remain unwritten.

The advancement of nutritional research and its application for the benefit of man were Mellanby's own chief scientific interests throughout his life. His interest in the subject was already aroused by the late Sir Frederick Gowland Hopkins who was one of his teachers at Cambridge; his early investigations of the effect of fat-soluble vitamins on calcification led to the discovery of vitamin D and hence to the abolition of rickets; some of his last experiments, on which he was engaged at the time of his death, were concerned with the analysis of the action of vitamin A, a subject to which he had continued to make notable contributions during the period of his official life. As Secretary of the Medical Research Council, and possessing as he did great personal scientific authority, he was in an unrivalled position to further the development of nutritional research and to ensure that its results were sensibly applied; he used the opportunity to the full, and the debt that Great Britain owes to him in this matter is too well known to need emphasis.

At the time of Mellanby's appointment as Secretary, the foundation of the Medical Research Council's activities had been laid, and its position as an important factor in the scientific life of the country had been established by the work of the late Sir Walter Fletcher; the organization remained nevertheless a small one and neither Mellanby nor anyone else could have foreseen the great expansion of the activities and responsibilities of the Council that was destined to occur during his secretaryship. At the same time it was clear that the range of work covered by the Council must be a wide one, and the news of Mellanby's appointment caused surprise and even misgiving among those who had regarded him as a man who was entirely devoted to personal research and destined to live out an academic career. In the event these doubts were completely dispelled by the way in which Mellanby rose to the demands of his new work, showing as he did so that, far from being narrowly confined in his interests, he had an unusual grasp of the problems of medical research as a whole.

In view of his own scientific enthusiasm it is not surprising that Mellanby accepted the appointment as Secretary of the Medical Research Council with the declared intention of continuing his personal researches and indeed on condition that he should be allowed to do so. His determination in this matter was so strong that even the inconvenience of weekly journeys to Sheffield was not allowed to prevent him from maintaining regular contact with the laboratory, and later, when a laboratory had been provided for him in London, however arduous his official duties might be, the week-ends were devoted to experimental work in which he seemed to find true relaxation. Nor was his attention to his laboratory in any way perfunctory; on the contrary he chose for study difficult problems that other people tended to avoid, and he produced results that made him in his own right an outstanding figure among medical research workers to the end of his life. A measure of his achievement is the fact that he was able in 1943 to give a memorable Croonian Lecture before the Royal Society on work that he had done during his period of office.

The more that one reflects upon Mellanby's powers as an investigator the more surprising it seems that, in his late forties, he should have been prepared to leave the academic groves for an official appointment. Certainly he already had a strong loyalty to the Medical Research Council, which was the body to which he was largely indebted for his own opportunity in research; certainly also he saw that the Council could exercise great influence over the development of medical research in Britain; probably he under-estimated the amount of work that the secretaryship would involve. Nevertheless he must have known that if he were to do his new job satisfactorily his main effort would be inevitably diverted from the laboratory which he loved. What then was the compelling motive?

The answer to this question lay in a side of his character that was not revealed until he assumed an official position. That he had persistence and determination was already evident; in addition there soon appeared other and unsuspected powers of judgement and vision. His judgement was manifested by his selection of the really important scientific projects and by his success in finding the right men to develop them; his vision by his flair for perceiving the administrative changes and developments that were required to further the cause of medical research. Moreover he acquired a high degree of confidence in his own clear ideas of what ought to be done; this confidence, growing with his own evident success, ultimately gave him a position of quite outstanding influence in the scientific and medical life of the country. There is no doubt that he enjoyed this position, and the fact that he came to do so itself throws some light on the reasons that must have influenced him to alter his career.

However much he may have become the powerful and successful scientific administrator, Mellanby always retained his sympathy for the research worker, perhaps particularly for the younger worker; nothing gave him greater pleasure than to hear from some young man working for the Medical Research Council of an interesting new discovery. This sympathy caused him to adhere firmly to the principle that in scientific administration the interests of the scientific workers must always come before those of the organization. His known convictions on this point gave confidence to all who worked for the Council, and indeed they are largely responsible for the spirit that pervades the organization; it would surely be a disaster for medical research in Britain if the principle to which Mellanby attached so much importance should be lost sight of.

Even Mellanby, with all his unusual powers, could not have accomplished what he did unless the circumstances of his life had been favourable. Of these circumstances there is one which mattered above all, as he himself well knew; he enjoyed a perfect partnership with a wife who, being herself a distinguished scientist in her own right, willingly sacrificed her personal interests so as to enable him to maintain his own scientific effort. When we reflect how much this effort influenced his official work and how much in turn this work has meant for Britain, we do well to remember that the debt is not to him alone.

No man of Mellanby's single-minded temperament and strength of character could be an easy man, and none even of his most ardent admirers would wish to deny that, like the rest of us, he had his failings, which for the most part were the counterpart of his great qualities. In scientific work his very eagerness for new discoveries and for their successful application in medicine certainly affected his judgement, and made it difficult for him at times to appreciate the efforts of those whose work, though sound, was not obviously productive; his passion for the laboratory prevented him from realizing the full possibilities of research at the bedside. In administration his perspicacity in larger issues was accompanied by a lack of interest in detail which caused him to underrate the value of the work that had to be carried out by others in order that his ideas might be brought to fruition; nor could he bear with patience those who did not immediately see things as clearly as he did himself. When he was preoccupied, especially with official matters, he could appear forbidding and unapproachable, and his peculiar technique of calculated provocation, whilst it often achieved the desired result of a vigorous and uninhibited response, could also, when applied to a diffident individual, stun and cause resentment. These failings unquestionably made personal relationships difficult at times, but the recollection of them will predominate only in the minds of those whose acquaintance with him was limited to official terms.

Such will indeed retain a very incomplete, if not distorted, picture of Mellanby. They may be left with respect for his obvious sincerity and ability, but they will never have seen his face light up at the news of an exciting piece of scientific work; they can never realize the warmth of the human sympathy that he could feel, especially for anyone who was to his knowledge making a real effort; most of all, they would have been astonished at the difference between Mellanby preoccupied with official matters and Mellanby in his own home or in other people's homes, when, having cast off his cares, he showed an almost boyish delight in simple pleasures.

We who were fortunate enough to know him in many circumstances shall remember his faults as well as his virtues, because we remember the whole man. But there is no doubt as to the impression that will fill our minds and will remain with us for the rest of our lives. It is the impression of a man with greatness in him: a great scientist and a great public servant; above all a man whose life was a shining example of uncompromising devotion to a fine ideal, the service of science to medicine.

Charles Harington

This symposium was planned by a Committee presided over by the late Sir Edward Mellanby: it now takes the form of a Memorial Number to him.

We are much indebted to the Scientific Editor, Professor B. S. Platt, for all the help he has given since Sir Edward's death.

THE WORK OF THE ACCESSORY FOOD FACTORS COMMITTEE

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MARGARET HUME M.A.

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1 Origin of the Committee: " The Newer Knowledge of Nutrition "
2 The post-war mission to Austria, 1919–22
3 International standardization of vitamins
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References

1. Origin of the Committee: " The Newer Knowledge of Nutrition "

Walter Morley Fletcher was the first Secretary of the Medical Research Committee (later Council), and the Accessory Food Factors Committee was the child of his wisdom. It was to be a "special investigation committee upon vitamines and accessory factors in metabolism " and was a small group appointed jointly by the Lister Institute of Preventive Medicine and the Medical Research Committee. It consisted of five members: Arthur Harden, Edward Mellanby and Jack Drummond, with Frederick Gowland Hopkins as Chairman and Harriette Chick as Secretary. As time went on the Committee was enlarged, and its subsequent Chairmen were Edward Mellanby and Rudolph Peters. The Committee had its first meeting on 6 May 1918. At first it was concerned chiefly with gaining and spreading information of " the newer knowledge of nutrition " as it was fitly named by E. V. McCollum (1919). Accessory food factors had recently been discovered; like minerals they provided no dietary calories, but were equally essential for correct nutrition.

The time was ripe for spreading the new knowledge, and there was a goodly pile to be spread. At the Lister Institute, London, work had proceeded for several years on the nutrient factor, situated in the outer coats of the rice grain, which was removed by modern methods of milling, but could protect the rice-eater from the nutritional disease of beriberi if retained in the diet. The work of Braddon and Cooper had confirmed the results obtained by Eijkman in Java and had extended his work in a careful and roughly quantitative study to locate the protective substance in foods other than rice. With the pigeon and hen as experimental animals, Cooper had determined approximately the daily dose of different foods needed to prevent and cure the polyneuritis induced by a diet of polished rice. At the same time Casimir Funk was busy in the Biochemical Department of the Lister Institute, preparing a concentrated extract of rice polishings, from which he hoped to isolate the pure substance named by him " vitamine ".

The problem had been attacked from another angle in attempts to rear young animals on diets composed of purified materials. As early as 1906 Hopkins had concluded that " no animal can live upon a mixture of pure protein, fat and carbohydrate, and even when the necessary inorganic material is carefully supplied, the animal still cannot flourish ". He it was who first used the term " accessory food factor ". His experiments lasted over many years, and the results were published in 1912 (Hopkins, 1912). Contemporary workers in the USA, at Baltimore, made similar attempts and showed that, in addition to proteins, fats, carbohydrates and minerals, two unidentified food factors were required. One, resembling the antiberiberi substance in its properties and distribution among foods, was named by McCollum and his colleagues " water-soluble B ". The second, contained in certain animal fats and oils and richly present in cod-liver oil and other fish-liver oils, was known as " fat-soluble A".

When the Accessory Food Factors Committee was formed, the First World War was already in its fourth year, and among British forces abroad much disease had occurred which it was suspected might be of nutritional origin. A form of beriberi had been recognized among Australian troops in the Middle East by Charles Martin, Director of the Lister Institute, when serving in the Australian Army Medical Corps. Scurvy had broken out in the forces in Mesopotamia, and the official issue of preserved lime-juice had proved ineffective against it. The workers at home in the Lister Institute were stimulated by these occurrences to make more exact studies of the properties of the antiberiberi and antiscorbutic vitamins, and of their quantitative distribution among foodstuffs. For scurvy, the work with guinea-pigs of the Norwegian investigator, Axel Holst, provided an experimental basis.

Rickets in children was strongly suspected of having a nutritional origin, and one school of thought felt confident that the fat-soluble A of McCollum, richly present in cod-liver oil, was the specific nutrient for prevention and cure. The nutritional theory gained strong support from the experimental work of Edward Mellanby with young dogs.

Thus, by the spring of 1918 there was much new and reliable information about accessory food factors, and the first concern of the newly formed Committee was to present the new knowledge in a monograph. In its early days the Committee met frequently to consider the various sections, which were drafted by the different members, and finally edited by Arthur Harden. The monograph of 100 odd pages was in proof by June 1919 and published soon afterwards under the title of " *Report on the present state of knowledge concerning accessory food factors* (*vitamines*) ". It had immediate success, and a second edition was soon called for; this was published in 1924 and comprised 171 pages. Nearly 10,000 copies of the first two editions were sold. A final and much enlarged monograph appeared eight years later under the title of " *Vitamins: a survey of present knowledge* " (Medical Research Council, 1932).

2. The Post-War Mission to Austria, 1919–22

In the spring of 1919 reports began to reach Britain of the severe food shortages suffered in eastern Europe during the later stages of the First World War and after its end. In Vienna, for example, conditions were reported to be serious,

and nutritional diseases to be rife. In infants and young children some degree of rickets was said to be almost universal, often leading to grave and permanent deformity, and osteomalacia was common among elderly adults of both sexes.

The idea was conceived of sending a mission to investigate the state of nutrition in Vienna and to ascertain whether specific diseases might be connected with lack of specific vitamins in the scanty food supplies. An important possibility thus offered itself for ascertaining whether the results of the experimental work with animals, gathered in the previous decade, might be applicable to the nutrition of human beings. Accordingly, in the autumn of 1919, the Accessory Food Factors Committee decided to send its Secretary to Vienna with Dr Elsie Jean Dalyell, a medically qualified colleague from Australia, working at the Lister Institute with a Beit Memorial Research Fellowship.

Food deprivation proved to be as severe as had been reported and nutritional disorders abounded. The scarcity of milk explained the frequency of scurvy and rickets in infants and young children. Hunger osteomalacia affected elderly adults of both sexes belonging to the poorest class. Keratomalacia leading to blindness was occasionally found in infants and children whose diet had been seriously deficient in fats.

The medical and scientific professions in central Europe had been cut off from foreign scientific journals for several years; the new discoveries about accessory food factors and vitamins were largely unknown to them, and were regarded with the greatest scepticism. Nevertheless, some leaders of the medical profession received the visitors with sympathy and encouraged them to put the new theories to practical tests in the Vienna hospitals and clinics. The results of experiments with animals soon proved to be fully applicable to corresponding human nutritional diseases, and Professor Clemens von Pirquet, the well-known Director of the Vienna University Kinderklinik, welcomed the mission in his own excellent hospital and, with his staff, generously collaborated in trials on suitable small patients in his own wards.

The Accessory Food Factors Committee considered the conditions for study reported by the preliminary reconnaissance to be so favourable that they planned an elaborate investigation, with additions to the British staff, to last if necessary two years, in order to elucidate the cause of rickets and the part played by diet. Even in the well-run wards of the Kinderklinik, some degree of rickets was invariably present after a stay of some months. Negative, untreated controls were, therefore, already there in plenty, and the task of the British and Austrian investigators was to test the effect of adding vitamins to the diet in the form of cod-liver oil. Abundant proof was obtained that cod-liver oil could prevent and cure rickets without any change in environment. At the same time the equally potent protective and curative action of sunlight was amply demonstrated. The resources of the Klinik's *x*-ray department and the services of its distinguished *x*-ray specialist, Dr Hans Wimberger, were an invaluable part of the hospitality offered, and one indispensable for satisfactory investigation of a disease like rickets, where clinical assessment may be highly misleading.

The work of the mission was described in various papers in German and English journals, and in *Studies of rickets in Vienna, 1919–22* as a report to the Accessory Food Factors Committee (Medical Research Council, 1923). The cost of the mission to Vienna was borne jointly by the Medical Research Council and the Lister Institute.

3. International Standardization of Vitamins

By 1925 quantitative work with vitamins, whether biochemical or nutritional, had become gravely compromised by lack of any consistent bases of comparison. Many workers had devised units of animal response to which their names were often given and which, once established, were difficult to change. Since the units were based on animal response, they were subject to the width of variation to which animal response is subject, and so could not reliably be used for comparison. Like other biological substances incapable of chemical or physical estimation, certain of the vitamins were thus ready for a standard substance to be established as yardstick, with a unit defined in terms of the activity of a fixed amount of the standard substance. In 1924 the Permanent Commission for Biological Standardisation was set up by the Health Organisation of the League of Nations, and much of the work came into the hands of the Medical Research Council's Department of Biological Standards, directed by Percival Hartley. The Accessory Food Factors Committee, when asked to see what could be done towards setting up standards for vitamins, considered that an attempt could be made for vitamins A, D, B, and C. Subcommittees were accordingly set up to deal with each of these four vitamins. Much preparatory work was done with international co-operation and, when the first International Conference for Vitamin Standardisation was called in 1931, it was possible to propose a standard for each vitamin. It is true that no pure substances were available, but large samples of material could be provided which were of uniform composition and whose stability appeared to be dependable. A crystalline sample of mixed carotenes and a specimen of cod-liver oil (the *United States Pharmacopeia* reference oil), a solution of irradiated ergosterol in oil, an adsorbate from an extract of rice polishings on acid clay, and a sample of citrated lemon-juice, were accepted as the respective international standards for the four vitamins, and international units were defined as the activity of designated weights of them.

The standards worked surprisingly well on the whole, but as research progressed to the isolation of purer substances, it was obvious that they could be improved. A second International Conference for Vitamin Standardisation was called for 1934, and the Accessory Food Factors Committee and its Subcommittees set to work again on the preparatory work, and organized international co-operation even more extensive than before. Pure crystalline β-carotene replaced the sample of mixed carotenes, and pure crystalline L-ascorbic acid the sample of citrated lemon-juice. The *United States Pharmacopeia* reference cod-liver oil was retained as subsidiary standard for vitamin A. No change was made in the standards for vitamin B_1 and vitamin D.

Spectrophotometric methods of measuring vitamin A had been greatly improved, but it was necessary to relate the readings thus obtained to the results of biological tests made against the international standard carotene and expressed in international units. The Vitamin A Subcommittee of the Accessory Food Factors Committee was concerned for many years with the value for this conversion factor and organized three crucial collaborative tests to determine it.

Between 1934 and 1939, pure esters of vitamin A were beginning to appear, and it became increasingly clear that vitamin A and not β-carotene ought to be the standard for vitamin A; also the *United States Pharmacopeia* reference

cod-liver oil had not been found wholly satisfactory. It occupied a somewhat enigmatic place in the complicated problem of the conversion factor, about the magnitude of which somewhat different views were held in the United Kingdom and the USA.

A third international conference was proposed for the end of 1939 at which it was planned to put forward crystalline vitamin B_1 as standard for that vitamin, DL-α-tocopheryl acetate as standard for vitamin E, and an ester of vitamin A as standard for vitamin A. The conversion factor for vitamin A was to be discussed again, and the possibility of using vitamin D_3 as standard for vitamin D was to be considered. The outbreak of the Second World War shattered the plans, and laboratories were evacuated with rats actually in the middle of biological tests.

The conversion factor became a lively practical issue during the war because concentrates of vitamin A for addition to margarine in Britain were bought in the USA. On the conversion factor depended their value in international units, and on their value in international units depended the price. Sellers liked a high value and buyers a low one, and the USA had always favoured a higher value than was accepted in Britain. Jack Drummond, when Scientific Adviser to the Ministry of Food, had a difficult time with the problem, which was eventually settled to the satisfaction of all in 1949 at an international conference called by the Subcommittee on Fat-Soluble Vitamins of the Expert Committee on Biological Standardization (World Health Organization, 1950). The same conference accepted vitamin A acetate as standard for vitamin A, and vitamin D_3 as standard for vitamin D.

Crystalline vitamin B_1 chloride hydrochloride and DL-α-tocopheryl acetate had already been established as standards for vitamin B_1 and vitamin E by correspondence during the war.

4. Work of the Committee in the Second World War

a. Composition of Wartime Bread

The work of the Accessory Food Factors Committee and its Subcommittees during the Second World War is described in *Medical research in war* (Medical Research Council, 1947). Among the Accessory Food Factors Committee's most important contributions were perhaps its two memoranda on the composition of wheat flour and bread (Medical Research Council, 1940, 1941).

It had been planned, apart altogether from the war, to fortify with vitamin B_1 the white, highly milled flour eaten by the majority, which contained about 73% of the wheat grain. Members of the Accessory Food Factors Committee were, however, of the opinion that such white flour lacked far more than vitamin B_1, and that the national health would be better maintained, especially in wartime, by milling away less of the outer parts of the wheat grain. They foresaw that if nutritional need did not compel such a change, difficulties of shipping space eventually would, so they busied themselves in working out what the specification for a national wheat flour ought to be. Invaluable help was given throughout by the Research Association of British Flour Millers. National bread, made from 85% extraction flour, relatively pale in colour but rich in protein and vitamins of the B complex, was the outcome of their deliberations and of experiments largely made in the Division of Nutrition of the Lister Institute. National bread remained the universal food of the people from 1942 till the end of the war and for some time afterwards.

It was not enough to set up the specification for National Flour. It was necessary also to find some means of enforcing adherence to the specification. A certain, stipulated content of vitamin B_1 and of fibre formed part of the specification, and these two characteristics were selected as criteria of adherence to the definition. Reliable and easy methods for use by public analysts in estimating vitamin B_1 and fibre were an indispensable part of the programme. Before the war the work of the Vitamin Subcommittees had been confined strictly to biological standardization. The war brought them other duties, and it was the Vitamin B Subcommittee of the Accessory Food Factors Committee to whom fell the difficult job of working out the method for estimating fibre, as well as a suitable chemical one for vitamin B_1 (Medical Research Council, 1943). In view of a possible shortage of calcium in the wartime diet, addition of calcium carbonate as *creta praeparata* to the National Flour was included in the specification.

b. Tables of Food Composition

The Ministries of Food and Health had many purposes for which they needed to know the composition of foods, but they soon realized that they were using different sets of tables, in which many of the values were hopelessly discrepant. The Ministry of Food turned for advice to the Medical Research Council. It was decided to prepare a new set of tables, and the Accessory Food Factors Committee deputed its Vitamin Subcommittees to deal with their respective vitamins in the matter. The results were put into use as quickly as they became available (Medical Research Council, 1945a).

c. Yeast as Human Food

The possibility that yeast might with advantage be introduced into the wartime diet as a source of extra protein and vitamins led the Accessory Food Factors Committee to initiate experiments with animals (pigs, rats) as well as human trials. The tests made with food yeast (*Torula utilis*) showed its special nutritive value as a supplement to diets composed largely of cereals (Medical Research Council, 1945b).

d. Human Requirement of Vitamin A and of Vitamin C

There was doubt as to the amounts of vitamin A and vitamin C that were really necessary for human health. The Ministry of Food had the responsibility for the national housekeeping and needed to know the minimum amounts per head of the population for which they must budget. Every bit of evidence had been marshalled that had become available since the Health Organisation of the League of Nations first took up the problem about 1935, but there was still wide uncertainty, and the Ministry made application to the Medical Research Council for guidance. The question was referred to the Accessory Food Factors Committee; they reported that only a human experiment could give the more exact information needed.

Fortunately the war provided an opportunity that could scarcely have occurred otherwise. At the Sorby Research Institute in Sheffield the Council was already maintaining about 20 conscientious objectors to military service who had volunteered to act as subjects for experiment. For almost two years some of them lived on a diet lacking vitamin A and carotene. During the period some signs of deficiency appeared which were sufficient to test the amount of vitamin A or of carotene which would cause them to disappear; at the

same time some volunteers were maintained free from signs of deficiency on minimum amounts of vitamin A or carotene. It was thus possible for the Accessory Food Factors Committee to make a definite recommendation about the requirement. Yet perhaps the revelation that in ordinary persons the liver reserves of the vitamin were large and were not depleted in less than a year or two was as important as any of the results (Medical Research Council, 1949).

The experiment with vitamin C followed that with vitamin A, and quite a number of the volunteers took part in both experiments. The second experiment was much more drastic than the first, and many of the heroic subjects developed indubitable scurvy which included for some of them an alarming heart condition. They subjected themselves also to experimental wounding. Happily there was no doubt about the nature of the condition, for it responded rapidly and specifically to administration of the vitamin. The knowledge obtained was exact and invaluable, and it was possible for the Accessory Food Factors Committee to reassure the authorities that the daily requirement for vitamin C was relatively small (30 mg.) and that, for instance, the USA assessment of the requirement (75 mg.) was far above the minimum (Medical Research Council, 1953).

Both experiments were directed throughout by the respective Vitamin Subcommittees, and they afford the interesting spectacle of a long and complicated experiment successfully conducted by a whole committee.

e. Prevention of Goitre

Surveys made early in the war in Oxfordshire, and reported to the Accessory Food Factors Committee, led to the formation of a Goitre Subcommittee in May 1943. Its activities included further surveys and led to the recommendation that " Prophylaxis against thyroid enlargement and goitre by the general use of iodized salt is desirable in Great Britain " (Murray, Ryle, Simpson & Wilson, 1948).

f. Milk Substitutes

After the Second World War as after the First, there was a shortage of milk in many parts of Europe. In Italy an infant food prepared from malted cereals and soya-bean meal had been used satisfactorily as a substitute. It was tested in the laboratories of the Lister Institute and was found to support the growth of rats.

There was a great shortage of milk in Germany too, and the Accessory Food Factors Committee, through a Cereals Research Subcommittee which it had set up, sponsored trials with infants carried out by the Medical Research Council's unit studying post-war undernutrition in Wuppertal. The success of the German trials led to more extensive researches in Africa and to the establishment by the Medical Research Council at Kampala in Uganda of a paediatric research group to study malnutrition in infants and young children, especially protein malnutrition which occurs in various forms and is so widely spread throughout the tropics.

* * *

The Accessory Food Factors Committee is still in being but there is less for it to do now that " the newer knowledge of nutrition " is no longer new. It is more than thirty years since the famous little book by McCollum (1919) was first published.

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VITAMINS AND THE PROTECTION OF THE LIVER

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1 Fatty livers, cirrhosis and other signs of choline deficiency
2 Discussion of lipotropic phenomena
3 The lipotropic agents (choline and its precursors)
4 Pharmacology of choline
References

We were happy to accept the suggestion of our friend the late Sir Edward Mellanby that we contribute an article on vitamins and the protection of the liver to this number of the Bulletin. While Sir Edward was never actively engaged in the study of lipotropic phenomena, his great breadth of knowledge in fundamental medical sciences was well illustrated by his familiarity with this field. The friendly stimulating criticisms and suggestions, made during his last visit to Toronto with Lady Mellanby in November 1951, are vividly remembered.

There do not seem to be any clear-cut gross effects of a deficiency of the water-soluble vitamins on the liver, with the outstanding exception of choline. This review will deal, therefore, with the role of choline and its dietary precursors, the so-called lipotropic agents, in the protection of the liver.

While there is no evidence that lack of vitamins A or D affects the structure or function of the liver, an acute lack of vitamin E leads in some species, at least, to liver necrosis. In the absence of vitamin E, an acid-fast, insoluble material, ceroid, with the staining characteristics of an altered lipid, accumulates in the liver of rats, especially when the diet contains unsaturated fatty acids. Ceroid is rarely seen in sections of tissue collected from human livers by biopsy or autopsy.

More exact studies of liver composition (especially with respect to nucleoproteins and enzymes) following dietary deficiencies may reveal hitherto unsuspected differences. A few communications dealing with this aspect of the subject have already appeared (see, for example, Campbell & Kosterlitz, 1948, 1950, 1952; see also Goldsmith, Unglaub & Gibbens, 1952; *Nutrition Reviews*, 1953; Sebrell & Harris, 1954).

1. Fatty Livers, Cirrhosis and Other Signs of Choline Deficiency

Choline was the first of the accessory food factors that was shown to protect the liver specifically, and to date it remains unique in this respect. Many species of animals develop abnormal accumulations of fat in the liver when diets low in choline are consumed. In some species the deposition becomes excessive (20% to 40% of fresh liver weight), but in others stainable fat appears although the total lipids present (estimated by exhaustive extraction with hot ethanol) may be only slightly above normal.

The term " fatty " as applied to the liver has been used by pathologists and biochemists with a somewhat different meaning. The pathologist describes a liver as fatty if fat is evident microscopically in suitably stained sections, even if certain regions only are affected. The chemist feels justified in describing the liver as fatty only when the extractable lipids are significantly beyond the normal range of values for the species. Stainable fat is sometimes seen in considerable amounts before there is any significant increase in the amount of lipid material extractable by suitable solvents. An erroneous impression may be obtained of the severity of fatty livers produced in different laboratories unless attention is paid to the base to which the analytical data are calculated. Some investigators express their results on a fresh-tissue basis, while others refer the data to dry tissue, others to fat-free tissue and some to dry, fat-free tissue. Typical data (Table I) taken at random from some of our experiments will illustrate the relationship between the values calculated in different ways.

TABLE I. APPROXIMATE RELATIONSHIPS BETWEEN PERCENTAGE VALUES FOR TOTAL LIVER LIPIDS CALCULATED TO FOUR DIFFERENT BASES

Fresh liver (%)	Fat-free liver (%)	Dry liver (%)	Dry, fat-free liver (%)
6.3*	6.7*	22.7*	29*
15†	18	42	75
30†	43	65	185
40†	67	76	305

* Average of 20 healthy young adult male rats from our colony.
† Values for livers with high lipid content.

When the diet lacks choline, stainable fat may be seen in the centrolobular cells of the rat and mouse within 24 to 48 hours after transfer from a normal to a severely hypolipotropic diet (Hartroft, 1950; Buckley & Hartroft, 1955). Fatty livers due to lack of choline or its precursors have been observed in the rat (Best, Hershey & Huntsman, 1932); mouse (Best, Huntsman & Solandt, 1932; Welch & Welch, 1938); dog (Best, Ferguson & Hershey, 1933; Chaikoff & Connor, 1940); rabbit (Blumberg, MacKenzie & Seligson, 1942); hamster (Handler & Bernheim, 1949); guinea-pig (Reid, 1954; Casselman & Williams, 1954); calf (Johnson, Mitchell, Pinkos & Morrill, 1951); pig (Neumann, Krider, James & Johnson, 1949); monkey (Mann, Andrus, McNally & Stare, 1953); duckling (Bernard & Demers, 1949). For reviews of early work see Griffith (1942), Lucas & Best (1943), McHenry & Patterson (1944), Jukes (1947), Best & Lucas (1950).

In the guinea-pig it is difficult to produce fatty livers of this type (Handler, 1949), apparently because it dislikes the unnatural rations. Reid (1954) showed that very young guinea-pigs will eat certain types of semi-purified diets and she reported the production of a clear-cut deficiency of choline. In our laboratory Casselman & Williams (1954) have shown that somewhat older guinea-pigs, fed purified diets lacking

choline, deposit many fine droplets of fat in the centrolobular hepatic cells although, as R. J. Young (unpublished data, 1955) has found, the extractable liver lipids are not increased significantly. The fatty livers produced in guinea-pigs fed scorbutic diets (Spellberg & Keeton, 1939) are probably not attributable to a lack of dietary choline. It must be kept in mind that there may be interference with the function of hepatic cells by deficiencies other than choline that may lead indirectly to fatty livers.

György & Goldblatt (1939) observed cirrhosis in rats, and Chaikoff & Connor (1940) in dogs maintained for long periods on choline-deficient diets; others have confirmed and extended these findings. Chronic choline deficiency in the rat leads to the development of various types of neoplasms in a significant percentage of the experimental animals (Copeland & Salmon, 1946; Engel, Copeland & Salmon, 1947; Schaefer, Copeland, Salmon & Hale, 1950). Cirrhosis from prolonged choline deficiency has also been reported in mice (Highman & Daft, 1951) and in rabbits (Rich & Hamilton, 1940). However, Wilson (1951) and Buckley & Hartroft (1955) obtained only minimal fibrosis in mice and observed development of neoplasms without cirrhosis. Mice fed on a semi-synthetic diet diluted with bentonite developed an unusual frequency of hepatomas (Wilson, 1953). This clay, which acts as a cationic exchange resin, adsorbs choline and other essential basic substances. Thus it is difficult to estimate to what extent choline deficiency alone is responsible for the neoplasms observed under these peculiar conditions. For excellent reviews of current knowledge in this field see " Nutritional Factors and Liver Diseases " (consulting editor, Schwarz, 1954).

The role of choline in human nutrition is less well known. It is possible that the alcoholic patient may exhibit symptoms of choline deficiency but, unfortunately, the picture here is complicated by the presence of lesions due to many other dietary deficiencies.

Choline deficiency leads to a number of other abnormalities. The haemorrhagic kidney syndrome was first observed in young rats by Griffith & Wade (1939). Somewhat similar renal lesions have been seen in young pigs (Neumann *et al.* 1949) and calves (Johnson *et al.* 1951). Hove, Copeland & Salmon (1954) have reported in rabbits kidney lesions that are not haemorrhagic but that can be prevented by choline. The failure to produce the haemorrhagic kidney lesion in young mice and puppies, both of which readily develop fatty livers, provides an interesting metabolic problem.

Some of the lesions found in choline deficiency are secondary to the renal lesion, e.g. the ocular lesions (Griffith & Wade, 1939; Bellows & Chinn, 1943) and hypertension (Hartroft & Best, 1949). It is less clear whether the cardiac and vascular lesions reported by Wilgram & Hartroft (1955) are the result of a direct or indirect effect of choline deficiency. Haemorrhagic lesions in weanling rats fed choline-deficient diets have been described in heart muscle and adrenals (Engel & Salmon, 1941). Other effects include involution of the thymus (Griffith & Wade, 1939; Christensen, 1940), muscular weakness in pigs (Neumann *et al.* 1949) and in rats (Aloisi & Bonetti, 1952), and muscular dystrophy in rabbits (Hove & Copeland, 1954). Skin lesions due to choline deficiency have been observed in rats (Wilgram, in preparation). Baby chicks, turkey poults and ducklings develop perosis (slipped tendon disease) and fail to grow on diets poor in choline and its precursors (Jukes & Almquist, 1942).

2. Discussion of Lipotropic Phenomena

Best, Huntsman & Ridout (1935) introduced the word " lipotropic " to describe substances that prevent (or decrease the rate of) deposition of abnormal lipids in the liver and that accelerate the removal of excess fat from it. Since the authors were discussing a nutritional situation, it was obvious that the word lipotropic referred to a dietary component and not to a hormone or to an environmental state. Subsequently, difficulties arose in deciding on the proper scope of the term. Sellers, Lucas & Best (1948) suggested inclusion of the other established effects of choline and its precursors, viz., prevention of renal lesions and of dietary cirrhosis. Wilgram, Hartroft & Best (1954a) further extended the term to include the prevention of the accumulation of abnormal (stainable) fat not only in the liver and kidneys but also in the heart and blood vessels of animals that exhibit those lesions when choline and its precursors are inadequate in the diet. This proposal was criticized by Mann (1954). As Wilgram, Hartroft & Best (1954b) have pointed out recently, Mann would probably not have objected to this proposal if these various manifestations of choline deficiency had been discovered and reported simultaneously.

It is important to recognize that a substance may appear to exert a lipotropic effect without being a lipotropic agent.

It might do this in several ways: for example, by increasing the rate of formation within the body of a lipotropic agent from dietary precursors or by potentiating in some way the action of such a compound. Many growth factors can alter the requirements for choline and methionine. Vitamin A, calcium, thiamine, biotin or any other dietary essential in whose absence the food intake and rate of growth are diminished may also affect the choline requirement. It would be unreasonable to call all the essential food factors antilipotropic agents. Restoration of a missing food factor to an inadequate diet will improve growth, and, unless extra choline is included in the improved ration, there will be a tendency for a fatty liver to develop if the previous diet was marginal with respect to lipotropic agents. The role of the antibiotics in lipotropic phenomena is still not entirely clear (György, 1954).

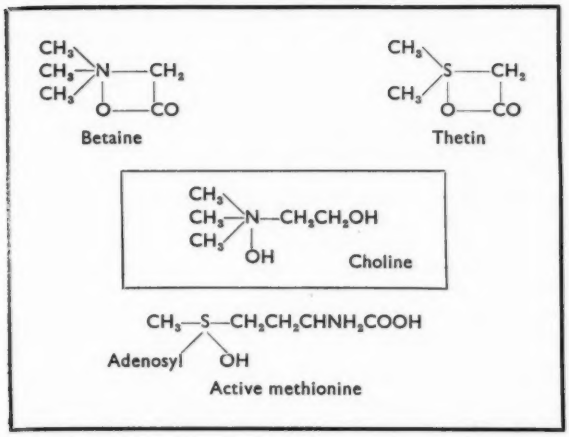
3. The Lipotropic Agents (Choline and its Precursors)

Choline, the key substance in lipotropic phenomena, was isolated from bile over one hundred years ago by Strecker (1849, 1862), but it was not until 1932 that it was recognized by Best, Hershey & Huntsman as a dietary essential. Its formula and relationship to some of the other compounds with lipotropic activity are shown in fig. 1.

Choline occurs ubiquitously in plant and animal materials, but rarely, if ever, in the free form. Some approximate values (as mg./g. of fresh substance) are as follows: egg yolk, 15–17; liver, 3–7; kidney, 1–3; lean meats, 0.4–1.2, usually about 0.9; fresh milk, 0.15; skim-milk powder, 1.6; wheat germ, 4; wheat and oats, 0.3–1.4, usually about 0.9; yellow corn (maize), 0.4; soya-bean meal, 2.8–3.5; peanut meal, 2–3. Fruit juices and vegetables are, in general, poor sources of choline. Refined edible fats and oils are essentially free from choline (Fletcher, Best & Solandt, 1935; Engel, 1943; Rhian, Evans & St John, 1943; McIntire, Schweigert & Elvehjem, 1944; Borglin, 1947).

The work of many investigators has established the fact that betaine, protein, methionine, thetins and—under some

FIG. 1. COMPOUNDS WITH LABILE METHYL GROUPS



conditions—vitamin B_{12}, folic acid and antibiotics can protect rats in varying degrees against the development of fatty livers of various types. Inositol offers some protection when diets free from fat are fed (Best, Lucas, Patterson & Ridout, 1951; Best, Ridout, Patterson & Lucas, 1951).

Betaine, methionine and thetins have been found to be dietary precursors of choline. Betaine occurs in many plants and is occasionally found in some animal tissues (Guggenheim, 1951). Methionine is present in practically all proteins but many plant proteins are poor in it (Block & Bolling, 1951). Arachin, the globulin of the ground-nut, is noted for its low methionine content, about 0.6% (Baernstein, 1932). The use of defatted ground-nut or soya-bean meal, or of other pulses, permits one to design hypolipotropic basal diets low in methionine and practically free from vitamin B_{12}. By extracting the meals with dilute ethanol (50%, v/v), followed by treatment with 80% (v/v) and finally with warm 95% (v/v) ethanol, a product almost free from choline and betaine may be obtained.

Several years ago it was questionable whether one should include the thetins in the list of dietary precursors of choline, but recent work indicates that they may be dietary sources of labile methyl groups for the biosynthesis of choline (Challenger & Simpson, 1947, 1948; Bywood, Challenger, Leaver & Whitaker, 1951; Challenger & Hayward, 1954; McRorie, Sutherland, Lewis, Barton, Glazener & Shive, 1954). Some years ago, Bennett (1941) showed that a thetin (the methyl sulphonium derivative of methionine) can replace methionine for growth, in the diet of rats.

Best & Huntsman (1932) reported that betaine has an effect on liver fat similar to that of choline. Soon it became apparent that the protein content of the diet also has a marked effect (Best & Huntsman, 1935; Channon & Wilkinson, 1935; Best, Grant & Ridout, 1936; Channon, Loach, Loizides, Manifold & Soliman, 1938). Channon and his colleagues embarked upon a systematic study of the amino acids to see which, if any, possessed lipotropic activity (Beeston & Channon, 1936; Beeston, Channon & Platt, 1937; Beeston & Platt, 1939). The effect of protein appeared to be accounted for when Tucker & Eckstein (1937) discovered that methionine possesses marked lipotropic activity. This finding was soon confirmed and extended in several laboratories (for literature see Lucas & Ridout, 1955). Beveridge, Lucas & O'Grady (1944, 1945) pointed out that the lipotropic activity of a protein is determined not only by its content of methionine and cystine but also by the nature and amount of the other essential amino acids contained in it. More recently a number of papers have emphasized the fact that dietary factors other than choline and its precursors affect the deposition of fat in the liver. Pertinent literature and discussion will be found in recent articles by Lucas & Ridout (1955) and Best, Hartroft, Lucas & Ridout (1955), who showed that a distinct lipotropic effect of casein may be observed in rats under conditions that appear to preclude any explanation involving the methionine or choline content of the diet. They observed that, in rats fed diets low in protein (9% or less) and containing a moderate amount of fat, the stainable fat appeared first in cells in the periportal regions of the liver lobules. This is in contrast to the initial appearance of stainable fat around the central vein when the fatty liver results from choline deficiency. Supplementary choline fails to prevent the accumulation of fat in periportal regions. A periportal distribution of fat was noted by Shils & Stewart (1954a, 1954b) in the livers of rats fed on corn meal, and they remarked upon the similarity of the lesion to that described by many clinicians in children suffering from kwashiorkor. Later, Shils, Friedland & Stewart (1954) observed a similar distribution in rats fed on other plant materials (e.g. rice, cassava and wheat flour). They found that lysine and tryptophan prevented the development of these fatty livers. The recent paper of Best *et al.* (1955) suggests that this type of fatty liver is typical of diets poor in protein, regardless of the source of the protein. They point out that protein deficiency may seriously interfere with the formation of many vital factors (such as nucleoproteins, lipoproteins, enzymes, etc.—cf. Campbell & Kosterlitz, 1948, 1950, 1952; Dick, Hall, Sydenstricker, McCollum & Bowles, 1952—) that the lack of choline would not be likely to affect to the same extent and that addition of supplementary choline could not, of course, correct.

In the same year that methionine was found to possess lipotropic activity, it was shown to be another essential amino acid (Womack, Kemmerer & Rose, 1937), and cystine lost this distinction. The consequent revival of interest in the sulphur-containing amino acids brought to mind the amino acid, homocystine, which had been produced some years earlier by treating methionine with hot, moderately strong sulphuric acid (Butz & du Vigneaud, 1932). When Professor W. C. Rose in Urbana and Professor du Vigneaud in New York tested the ability of homocystine to replace methionine in the diets of rats, they got diametrically opposite results; the rats in Urbana grew (Rose & Rice, 1939), those in New York died—with fatty livers (du Vigneaud, Dyer & Kies, 1939). The different responses were traced to a difference in the vitamin supplements provided. The fatty livers suggested a lack of choline in the diets supplied by du Vigneaud and his associates. Choline was shown to be present in the tikitiki concentrate offered in Rose's laboratory, and addition of supplementary choline or betaine to the diets fed in New York produced growth. The fact that choline and betaine contain methyl groups suggested the possibility that the rat is able to transfer a methyl group from these compounds to homocysteine and effect a biosynthesis of methionine. Thus du Vigneaud, Chandler, Moyer & Keppel (1939) were led to

postulate a new type of biochemical process—transmethylation. Subsequent studies with choline, betaine, methionine and other compounds containing deuterium-labelled methyl groups established the reality of this exchange. One must differentiate between the " biologically labile " methyl groups of choline, betaine and methionine on the one hand, and the non-labile methyl groups of sarcosine, creatine and of purines and pyrimidines on the other. Transmethylation reactions were shown to occur in other species: in man by Simmonds & du Vigneaud (1942), and in the rabbit by Schenck, Simmonds, Cohn, Stevens & du Vigneaud (1943).

The concept of transmethylation, by implication, led to the idea that the animal organism is unable to produce methyl groups by synthesis *de novo*, and in most of the earlier experiments there seemed little reason to doubt this. Later it was possible to tag the methyl groups with radioactive carbon (^{14}C). By mixing methionine so labelled with deuterio-L-methionine, a doubly labelled product was obtained (Keller, Rachele & du Vigneaud, 1949). An excellent review of this field may be found in the book, *A trail of research* (du Vigneaud, 1952).

Double labelling has revealed that two types of methylation occur in the body: (i) there may be transfer of an intact methyl group, or (ii) some one-carbon fragment may be reduced to form a new methyl group. The first process is correctly described as transmethylation, the second involves biosynthesis of a new methyl group from some dietary or metabolic precursor (Arnstein, 1950, 1951; Sakami & Welch, 1950; Sakami, 1950). The one-carbon fragment that acts as immediate precursor appears to be either formate or some active unit containing the formyl radical. This is not the place to review in detail the brilliant tracer studies of Elwyn and Sprinson and their colleagues, of Arnstein and Neuberger, of Sakami and Welch and of many others who have contributed to our present knowledge in this field. In many experimental situations the data fail to provide unequivocal differentiation between the two methods of biological methylation. Later it was established that the methyl groups of choline are not biologically labile, as was at first believed (Dubnoff, 1949). Choline must first be oxidized to betaine. One of the methyl groups of betaine is labile in transmethylation reactions (cf. Muntz, 1950). One or more of the methyl groups of thetins are also labile under the influence of appropriate enzymes. The enzymes concerned in these reactions appear to be specific for each donor-acceptor pair.

About five years ago it seemed that all the compounds serving as methyl donors were methylated onium compounds, with the notable exception of methionine. Then it developed that methionine had first to be converted to an active form, by the presence of adenosine triphosphate. "Active methionine " was found to be free from phosphorus and now has been identified by Cantoni (1952, 1953) as an *S*-adenosyl addition product. Thus " active methionine ", like all the other donors of labile methyl groups, has proved to be an onium compound. The role of onium compounds as high energy substances has been discussed by Woolley (1953).

In 1943 Toennies, Bennett & Medes obtained growth (slow it is true, but continued) in rats fed diets containing homocystine but no choline, betaine, methionine or other known source of methyl groups. This observation and subsequent studies (Bennett, Medes & Toennies, 1944) revealed that the rat, and presumably other animals, can to a limited degree make methyl groups, but only when the diet contains certain essentials. The possibility that intestinal bacteria are responsible for the synthesis of new methyl groups was eliminated by repeating some of the studies on germ-free rats at the University of Notre Dame, Indiana, USA (du Vigneaud, Ressler, Rachele, Reyniers & Luckey, 1951).

The pair of vitamins that are of such importance in haematopoiesis, viz., folic acid and vitamin B_{12}, have been shown to play a part also in lipotropic phenomena. The picture is by no means entirely clear, but most of the experimental findings are consistent with the view that vitamin B_{12} is involved in the biosynthesis of new methyl groups but is not concerned in transmethylation (Stekol, Hsu, Weiss & Smith, 1953; Arnstein & Neuberger, 1953). Evidence that folic acid is involved in the metabolism of the one-carbon fragment was supplied by Elwyn & Sprinson (1950), Plaut, Betheil & Lardy (1950), Verly, Kinney & du Vigneaud (1952), Stekol, Weiss & Weiss (1952) and Elwyn, Weissbach, Henry & Sprinson (1955), but its role in lipotropic phenomena has not yet been fully elucidated.

4. Pharmacology of Choline

The amount of choline necessary to protect the liver has been found to vary with the age and sex of the animal, with the nature of the basal diet (Griffith, 1941) and with the environmental temperature (Sellers & You, 1952). Since the choline requirement appears to be related to the food intake it is usually expressed as percentage of the diet rather than as mg./day. Several dose-response curves for choline are available in the literature (Welch & Welch, 1938; Griffith & Mulford, 1941; Best, Lucas, Ridout & Patterson, 1950). The choline requirement of young adult rats varies from about 0.08% to 0.16%.

The choline requirement of man is not known. We have estimated that in Toronto the daily intake of adults on average mixed diets varies from 250 mg. to 600 mg., figures in good agreement with the calculated values of 300 mg. to 500 mg. reported by Borglin (1947) in Sweden. Johnson, Hamilton & Mitchell (1945) give rather lower figures (about 150 mg.) for young men in the USA.

The acute toxicity of choline, especially by mouth, is relatively low (about 5 g./kg. for rats) in comparison with that of some of its esters and of many other quaternary ammonium compounds (Mott & Halliburton, 1899, see p. 223; Hodge & Goldstein, 1942; Neuman & Hodge, 1945; Hodge, 1945; McArthur & Lucas, 1950). Further data may be found in *The vitamins* (Sebrell & Harris, 1954).

There is evidence that sometimes in rats and in human patients a considerable proportion of choline taken by mouth may be converted to trimethylamine and its oxide by intestinal bacteria before absorption can take place (Popper, de la Huerga & Koch-Weser, 1952; de la Huerga, György, Waldstein, Katz & Popper, 1953). However, Riedesel & Hines (1953) conclude that choline is absorbed unchanged from the small intestine of the rat and that intestinal bacteria play only a minor role, if any, in its disappearance.

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RECENT WORK ON B VITAMINS IN THE BLOOD AND GASTROINTESTINAL TRACT, ESPECIALLY IN RELATION TO HUMAN DISEASES

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1 Vitamins B_1, B_2, B_6 and nicotinic acid
2 Folic acid
3 Vitamin B_{12}
4 Relation between folic acid and vitamin B_{12}
References

The term " recent " will be taken to imply work reported in the last decade, much of it in the last quinquennium, and the scope of this paper will be restricted to work done on man. This will mean omitting further reference to the fat-soluble vitamins on which there has been no relevant work of importance in the period under review. Recent advances come mainly from three directions. The first is the analysis of the symptoms observed in men and women subjected to malnutrition and starvation in the Second World War. The second is the introduction of vitamin analogues or antagonists which permit studies of single deficiencies of vitamins that are not easily excluded from the diet. The third is the isolation of folic acid and vitamin B_{12}. This third advance has been the most important for the student of human disease, and the greater part of this article will be devoted to an account of it.

1. Vitamins B_1, B_2, B_6 and Nicotinic Acid

The effects of dietary deficiencies on the oral structures were fully reviewed by Schour & Massler (1945). Thiamine (vitamin B_1) had no specific effect but increased the sensitivity of the oral tissues. Riboflavin (vitamin B_2) deficiency was believed to be characterized by cheilosis and a magenta-coloured glossitis, in addition to corneal injection and seborrhoeic dermatitis. Nicotinic acid deficiency was characterized by a fiery glossitis, gingivitis and stomatitis, in addition to weakness, lassitude, insomnia, anorexia and loss of weight. It was not definitely established that oral symptoms were produced by deficiency of pyridoxine (vitamin B_6) or pantothenic acid. Observations during the war were in keeping with Schour and Massler's review, but the effect of later experimental work has been to diminish the importance of riboflavin and increase that of pyridoxine.

In the deficiency diseases in Japanese prison camps (Smith & Woodruff, 1951), the orogenital syndrome appeared to be closely correlated with the estimated riboflavin content of the diet and it was regarded as a specific manifestation of riboflavin deficiency. The orogenital syndrome is characterized by angular stomatitis, cheilosis, glossitis and pharyngitis, together with lesions of other mucocutaneous junctions and scrotal or vulvar dermatitis. Pellagra showed a fairly close correlation with the nicotinic acid content of the diet, and there was apparent aggravation by maize. Nutritional macrocytic anaemia was relatively uncommon and it particularly affected the pregnant woman. It responded to yeast extracts and to crude, but not refined, liver extracts, and in retrospect would appear to have been due to lack of folic acid.

The relation of deficiency of nicotinic acid to pellagra is established, but it is now realized that the tryptophan content of the diet is almost equally important. Goldsmith, Sarett, Register & Gibbens (1952) induced pellagra in three subjects who remained on a diet low in nicotinic acid and tryptophan for more than 50 days. Glossitis, stomatitis and cheilosis occurred, together with the other symptoms of pellagra. In experimental riboflavin deficiency induced by dietary depletion (Hills, Liebert, Steinberg & Horwitt, 1951), the alimentary lesions were not striking and were much less common than scrotal dermatitis. A few patients developed angular stomatitis, but glossitis was not observed.

It has not so far proved possible to produce a deficiency of pyridoxine (vitamin B_6) by dietary means alone (Hawkins & Barsky, 1948). However, Mueller and his co-workers (Mueller & Vilter, 1950; Vilter, Mueller, Glazer, Jarrold, Abraham,

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Thompson & Hawkins, 1953) induced pyridoxine deficiency in human patients by the combination of a diet poor in the vitamin B complex with the antagonist deoxypyridoxine. Glossitis, stomatitis and cheilosis similar to those due to deficiency of nicotinic acid and riboflavin were produced, but they responded only to pyridoxine. It is now evident that deficiency of a number of members of the vitamin B complex, as well as of the metal iron, can produce glossitis, stomatitis, cheilosis, gastritis and other alimentary symptoms which are similar in the different deficiencies. The symptoms probably have a common denominator in the chain of enzyme reactions that is concerned with the metabolism and replacement of rapidly multiplying cells. Rapidity of replacement of cells (Leblond & Stevens, 1948) is a factor common to gastrointestinal tissues and to blood, and it is this which provides part of the explanation of why alimentary and haematological symptoms are often associated together.

2. Folic Acid

Undoubtedly the major advance in haematology in the last decade has been the isolation and application of folic acid and vitamin B_{12}. The pigments of the butterfly's wing, now known to be pteridins, were one of the earliest objects of study of the late Sir Frederick Gowland Hopkins, who was a pioneer in the discovery of the vitamins. From 1940 onwards much interest was taken in the vitamin or group of vitamins known as folic acid. This was isolated from the liver in crystalline form by Stokstad (1943), shown to be pteroylglutamic acid and therefore related to Hopkins' pteridins, and finally synthesized (Angier *et al.* 1945). Folic acid was shown to repair the anaemia of all forms of megaloblastic degeneration of the bone marrow. It was also shown to relieve both the anaemia and the intestinal symptoms of tropical sprue, though it was less successful in non-tropical sprue and coeliac disease (Darby & Jones, 1945; Davidson, Girdwood & Innes, 1947).

It soon became clear, however, that folic acid was not the anti-pernicious anaemia factor, because it does not relieve, and indeed may exacerbate, subacute combined degeneration of the spinal cord. The lingual manifestations of pernicious anaemia may respond well or poorly to folic acid, and they may relapse after a period of relief (Schieve & Rundles, 1949). Moreover, folic acid eventually ceases to control the anaemia of classical Addison's anaemia (Wilkinson, 1948); this is presumably because both vitamin B_{12} and folic acid are concerned in blood formation and neither can completely substitute for the other (Tasker, 1954).

In the body, folic acid is converted into a formyl derivative known as folinic acid or citrovorum factor, which is the physiologically active form. The conversion of folic acid to folinic acid is catalysed by ascorbic acid (Nichol & Welch, 1950), and May and his co-workers (May, Nelson & Salmon, 1949; May, Hamilton & Stewart, 1952) have produced megaloblastic anaemia in monkeys by feeding them on a diet poor in folic acid and ascorbic acid. Treatment with either folinic acid or ascorbic acid restored the marrow to normal and caused the accumulation of folinic acid in the liver. A similar mechanism is probably responsible for the megaloblastic anaemia that occasionally occurs in human infants and it may play some part in the megaloblastic anaemia of pregnancy. With these exceptions it may be said that human disease is rarely due to a failure in the conversion of folic acid to folinic acid. Spray & Witts (1952a) found no evidence of failure to convert folic acid to folinic acid in pernicious anaemia, and the two substances are equivalent in their therapeutic effect on pernicious anaemia (Davidson & Girdwood, 1951; Boscott & Cooke, 1954).

Assessment of Folic Acid Status

To clarify the relation between folic acid and vitamin B_{12}, efforts have been made to determine the folic acid status of the organism in different anaemias. Folic acid is assayed microbiologically, the test organisms usually being *Streptococcus faecalis* or *Lactobacillus casei*; for folinic acid *Leuconostoc citrovorum* is used. Unfortunately, folic and folinic acids have a low renal threshold and therefore determination of plasma levels is of no value. Spray & Witts (1953) showed that folinic acid was rapidly cleared from the plasma after intravenous injection, and the difference between normal people and patients with anaemia was small. Whole-blood levels of folic acid have been used by Nieweg and his coworkers (Nieweg, Faber, de Vries & Kroese, 1954), but others have not been satisfied with this method. Usually some type of saturation test has been employed.

Spray, Fourman & Witts (1951) found that when small doses of folic acid, 200–400 μg., were given intravenously on successive days, the excretion of folic acid in the urine was lower in pernicious anaemia than in normal subjects, and much lower in steatorrhoea. Spray & Witts (1952b) subsequently found that, when folic acid was given by mouth in a dose of 1 mg., there was a much smaller increase in the concentration of folic acid in the plasma, and a much smaller excretion in the urine, in untreated pernicious anaemia and steatorrhoea than in health. Girdwood (1953, 1955) has standardized a test in which the urinary excretion of folic acid after a dose of 5 mg. by subcutaneous injection is compared with the excretion after 5 mg. by mouth. In pernicious anaemia there is apparent tissue depletion of folic acid, but normal absorption. In steatorrhoea there is failure to absorb folic acid. In pernicious anaemia of pregnancy there is no evidence of tissue depletion or defective absorption of folic acid.

3. Vitamin B_{12}

Vitamin B_{12} was isolated in 1948, and the dosage and methods of administration were rapidly worked out by Ungley (1949a, 1949b) and others. Despite statements to the contrary by Owren (1950), the parenteral administration of vitamin B_{12} is now believed to provide a complete substitution therapy for pernicious anaemia. The marrow reverts from the megaloblastic to the normoblastic type of erythropoiesis, the blood count returns to normal, peripheral neuritis is cured, subacute combined degeneration of the cord is arrested, glossitis heals and the megaloid change which affects the epithelial cells of the gastrointestinal tract disappears (Graham & Rheault, 1954). Nevertheless, gastric atrophy and achlorhydria persist unchanged. The amount of vitamin B_{12} required for maintenance in good health is 1 μg. or less a day. The most economical form of therapy is to begin treatment with doses of 50 μg. at three-day intervals and gradually increase the interval between doses to two or three weeks. As the stores of vitamin B_{12} are completely empty in pernicious anaemia there may be advantages in beginning treatment with five daily injections of 1,000 μg., though much of this will be lost in the urine.

The isolation of vitamin B_{12} was accompanied by the discovery of methods of microbiological assay, the most satisfactory test organism at first being the alga *Euglena* (Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin & Jukes, 1949). Using this organism, Mollin & Ross (1952, 1953a, 1953b; also Ross, Mollin, Cox & Ungley, 1954) made simultaneous estimation of the level of vitamin B_{12} in the serum and urine and of the haematological response following administration of vitamin B_{12}. They found that most of the vitamin B_{12} in serum is present in a bound form, but a small proportion is free. It can all be converted to the free form by heating at 100°C. for 15 min. in an acid medium. The probable function of the binding material in the serum is to maintain a balance between vitamin B_{12} in the tissues and the blood. In normal subjects the total content of vitamin B_{12} may be between 100 and 720 μμg./ml., the average being 358 μμg./ml. In patients with pernicious anaemia during a relapse, however, it is much lower, between 16 μμg. and 105 μμg./ml., with an average of 40 μμg./ml., and is all bound. The critical level for vitamin B_{12} deficiency appears to be about 100 μμg./ml., and the serum level bears a definite relationship to haemopoiesis; above 100 μμg./ml. the marrow is normoblastic, and below this level it is megaloblastic. The serum concentration is lower if the anaemia is severe. The daily urinary excretion in normal subjects is 0.11–0.27 μg., while in pernicious anaemia during relapse it is slightly lower, from 0.02 to 0.15 μg.

Primary deficiency of vitamin B_{12} is rare, but a few instances have been observed in strict vegetarians who take no dairy products and are known as vegans (Badenoch, 1954; Wokes, Badenoch & Sinclair, 1955). Deficiency of vitamin B_{12} is nearly always secondary to disease of the alimentary tract. Mollin & Ross (1954) found that the serum vitamin B_{12} was always low in pernicious anaemia and in the megaloblastic anaemia of total gastrectomy. It was usually low in the megaloblastic anaemia of partial gastrectomy and of intestinal anastomosis or stricture. On the other hand, it was nearly always normal in pernicious anaemia of pregnancy, and more often than not it was normal in the megaloblastic anaemia of steatorrhoea. The observations of Mollin and Ross have been confirmed but hardly extended by a number of workers, most of whom have used other test organisms for microbiological assay, such as *Lactobacillus leichmannii* (Girdwood, 1954; Glass, Lillick & Boyd, 1954; Heinrich, 1954; Lear, Harris, Castle & Fleming, 1954; Pitney & Beard, 1954).

It is possible to prepare radioactive vitamin B_{12} of high activity by growing the *Streptomyces griseus* in a medium which has been fortified with a salt of radioactive cobalt, usually the isotope ^{60}Co, which has a half-life of 5.3 years; but more recently the isotope ^{58}Co, with a half-life of 72 days, has been used (Bradley, Lester Smith, Baker & Mollin, 1954). When this material is injected intramuscularly in man, it is found that the vitamin or its radioactive metabolites are concentrated in the marrow within four hours. Large concentrations of the vitamin are retained in the tissues of the body in the descending order: liver, spleen, sternum and kidney, and they persist there for at least two months after injection and probably much longer (Hall, 1954; Glass, Boyd & Gellin, 1955). The main use of radioactive vitamin B_{12} has been to study the absorption of the vitamin from the alimentary tract.

Absorption of Vitamin B_{12}

Castle (1929) first showed that the diet contains an extrinsic factor, now known to be vitamin B_{12}, which cannot be absorbed without the aid of an intrinsic factor in the gastric secretion. In the words of Castle (1953), pernicious anaemia " would not develop if the patient could effect daily the transfer of a millionth of a gram of vitamin B_{12} the distance of a small fraction of a millimeter across the intestinal mucosa and into the blood stream ". Intrinsic factor catalyses the absorption of vitamin B_{12}; it is believed to be a mucoprotein and it has been prepared in relatively pure and concentrated forms (Glass, 1953; Latner, Merrills & Raine, 1954; O'Brien, Taylor, Turnbull & Witts, 1955).

Heinle and his colleagues (Heinle, Welch, Scharf, Meacham & Prusoff, 1952) first used ^{60}Co-labelled vitamin B_{12} to estimate the absorption of the vitamin and thus to determine the presence or absence of intrinsic factor. The most accurate results are obtained when the dose of vitamin B_{12} is small—0.5 μg.—and the specific activity is high. In health about 70% of such a dose is absorbed, whereas in pernicious anaemia only 10% or less is absorbed (Callender, Turnbull & Wakisaka, 1954a). Patients who have undergone total gastrectomy behave like patients with pernicious anaemia, and in both groups absorption of vitamin B_{12} can be restored to normal if it is given together with normal human gastric juice or some other source of intrinsic factor (Callender, Turnbull & Wakisaka, 1954b). If a large dose of unlabelled vitamin B_{12} is injected shortly after a tracer dose of ^{60}Co-labelled vitamin B_{12} has been given by mouth, some of the radioactivity absorbed appears in the urine (Schilling, 1953). The urine test is a convenient method of measuring the absorption of vitamin B_{12}, but it is not as reliable as the determination of the amount of radioactive material in the faeces (Callender & Evans, 1955). A further development of the method is to measure the hepatic uptake of ^{60}Co-labelled vitamin B_{12} after an oral dose, by using a scintillation counter over the skin projection of the liver area (Glass, Boyd & Stephanson, 1954).

In steatorrhoea absorption of vitamin B_{12} may be normal, it may be as seriously impaired as in pernicious anaemia or it may lie between these extremes. If it is abnormal, there is rarely much improvement after administration of amounts of intrinsic factor effective in pernicious anaemia, though in some patients very large doses of intrinsic factor may bring absorption back nearly to the normal range (Evans, personal communication, 1955). In the rare instances of megaloblastic anaemia after partial gastrectomy and gastroenterostomy, the findings are usually identical with those in classical pernicious anaemia, but occasionally they are similar to those of idiopathic steatorrhoea (Witts, 1955; Badenoch, Evans, Richards & Witts, 1955b). In the megaloblastic anaemia of pregnancy and the puerperium, which occurs in about one in 1,000 pregnancies in Great Britain, the absorption of vitamin B_{12} is normal (Badenoch, Callender, Evans, Turnbull & Witts, 1955), as is that of folic acid (Girdwood, 1955), and the anaemia appears to be due to the development of resistance to the haemopoietic factors.

The absorption of physiological amounts of vitamin B_{12} depends on intrinsic factor, but detectable rises occur in the vitamin B_{12} of the serum and urine when large doses, of the order of 1,000 μg., are given by mouth without intrinsic factor in pernicious anaemia (Ross *et al.* 1954). Haematological effects have been observed when smaller doses, of the order of

50 μg., have been given daily to the fasting patient (Chalmers & Hall, 1954). Vitamin B_{12} is also absorbed when it is given by insufflation or inhalation (Monto, Rebuck & Brennan, 1953; Monto & Howell, 1955; Israels & Shubert, 1954). It is thus clear that vitamin B_{12} in high concentration can be absorbed through epithelia unaided, but for the absorption of the minute amounts present in the diet the special mechanism of the intrinsic factor is necessary.

4. Relation between Folic Acid and Vitamin B_{12}

The relation between folic acid and vitamin B_{12} is still obscure. The megaloblastic anaemias can be arranged in a continuous series: at one end Addisonian pernicious anaemia which responds completely to vitamin B_{12}, at the other end pernicious anaemia of pregnancy which is highly resistant (Moore, Lillie & Gatenby, 1955). All respond well to folic acid, provided the organism has not been completely denuded of vitamin B_{12}. In experiments on bone-marrow cultures, folic acid and folinic acid have been found to act directly on megaloblasts, producing ripening of these cells to normoblasts. Vitamin B_{12} alone does not ripen megaloblasts into normoblasts but it will do so if it is combined with normal gastric juice (Lajtha, 1950; Callender & Lajtha, 1951a, 1951b; Thompson, 1950). These findings have been explained by the theory that vitamin B_{12} requires intrinsic factor—or some similar substance derived from the tissues when vitamin B_{12} has been given parenterally—to enable it to pass into cells. On the other hand, Horrigan and his co-workers (Horrigan, Jarrold & Vilter, 1951) got directly contrary results when they injected vitamin B_{12} and folic acid directly into the marrow in man; vitamin B_{12} promoted the local maturation of megaloblasts into normoblasts, and folic acid did not. It should be noted that cytological studies of bone-marrow, both in vitro and in vivo, have a large margin of error. All that can be said at present is that both folic acid and vitamin B_{12} are necessary for erythropoiesis and that folic acid can largely but not entirely replace vitamin B_{12}.

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VITAMINS AND THE NERVOUS SYSTEM

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1 Metabolism of neurones
2 Thiamine
3 Pantothenic acid
4 Other hyperpyruvaemic neuropathies
5 Vitamin B_{12}
6 Pyridoxine
7 Nicotinic acid, riboflavin and other members of the vitamin B complex
References

Twenty-one years ago Sir Edward Mellanby wrote as follows:

> The field of nutrition has constantly been handicapped because much of the work has been in the hands of narrow specialists—biochemists with little or no biological or medical knowledge, and medical men with little or no fundamental biochemical or physiological training. By anybody with a wider grasp of physiological, biochemical, pathological and clinical subjects, a rich harvest will be gathered [Mellanby, 1934].

He stressed in this book, *Nutrition and disease*, the importance of combined research by clinical and laboratory methods, and urged the need "... to survey the field, pick out the best lines of attack on any particular problem, [and] keep in touch with the literature ..." The book illustrates his own experience of nutrition and disease in the fields of rickets, dental structure, the thyroid, infection, and in particular diseases of the nervous system, the last of these subjects also being incorporated in his Linacre Lecture, "The nervous system within the pale of nutrition". Sir Edward later acknowledged that although he was convinced that his approach to the problem of nutritional disorders of the nervous system was correct—and no one would doubt this—he had been led into erroneous interpretations for two reasons: first, it is dangerous to argue too readily from work on experimental animals to human diseases, and secondly he had not had time fully to survey the literature. Had he known that disseminated sclerosis is almost unknown in India and China, where deficiency of vitamin A is relatively common, he would not have written: "My interpretation of these facts is that the symptoms associated with early inflammatory changes of disseminated sclerosis are largely healed up and prevented from spreading [by dietetic treatment]". Similarly he would not have attributed "the actual polyneuritic change of beriberi" and "the nervous lesions of pellagra" to deficiency of vitamin A. But he saw the dire need for further work to elucidate these challenging claims:

> After all, the experimental conditions obtained by diets deficient in vitamin A and carotene represent the only method so far known of producing nerve lesions of close similarity to those found in convulsive ergotism, pellagra, lathyrism and subacute combined degeneration and until they can be produced by other means, it can only be assumed that vitamin A and carotene deficiency in the diet or ineffectiveness after absorption play an important part in their aetiology.

That was written in 1934. Twenty-one years later we fortunately have other means, and this is due in no small measure to the stimulus of Mellanby.

1. Metabolism of Neurones

Since the pioneer researches of Thudichum (1884) great progress has been made in the chemical constitution of nervous tissue and in its metabolism. Speaking generally, we can say that neurones, unlike other cells, rely for their energy almost exclusively upon the oxidation of carbohydrate; they appear unable to utilize fatty acids as a source of energy. The brain has a respiratory quotient of unity (Gibbs, Lennox, Nims & Gibbs, 1942), and it is profoundly affected by hypoglycaemia, since its glycogen is very stable and hardly affected by fasting or by feeding glucose. The brain's consumption of oxygen rises with increased activity but, in both the brain and peripheral nerve, energy is required also for trophic purposes, including the maintenance of structure. When therefore the axon is separated from the cell body or when the energy supply of the cell body is seriously impaired, axonal degeneration occurs. Glucose is used faster by brain slices than by those of liver, heart or kidney, but it is not the only aliment oxidized by brain: other hexoses and hexose phosphates are used, though less effectively, and lactate and pyruvate are quickly oxidized in vitro. But administration of the last two, unlike that of glucose, hardly affects the course of insulin hypoglycaemia, probably because they pass slowly from blood to brain; similarly, they have no restorative effect upon the brain when administered after hepatectomy.

The main pathway of degradation of glucose in brain is through the Embden and Krebs cycles. This has been elucidated largely by the work of the following: Lohmann (1933); Banga, Ochoa & Peters (1939); Cori & Cori (1940); Ochoa (1941); Racker & Krimsky (1948); and Utter (1950). The steps are therefore:

glycogen $\rightleftharpoons$ glucose-1-phosphate $\longrightarrow$ glucose-6-phosphate $\longrightarrow$ fructose-6-phosphate $\longrightarrow$ fructose-1 : 6-diphosphate $\longrightarrow$ glyceraldehyde-3-phosphate $\longrightarrow$ 1 : 3-diphosphoglycerate $\longrightarrow$ 3-phosphoglycerate $\longrightarrow$ 2-phosphoglycerate $\longrightarrow$ phosphopyruvate $\rightleftharpoons$ pyruvate $\longrightarrow$ acetyl-CoA[1] $\longrightarrow$ tricarboxylic acid cycle.

lactate $\uparrow\downarrow$ pyruvate

The following vitamins are concerned in these conversions: thiamine and pantothenic acid (for conversion, together with lipoic acid, of pyruvate to acetyl-CoA), nicotinic acid as part of DPN, and riboflavin as part of certain dehydrogenases. It might therefore be supposed that deficiency of any of these three vitamins might be followed by defective function of neurones, and such is the case.

Deproteinized extracts of brain contain relatively very large amounts of glutamic acid and of creatine. Other nitrogenous compounds in slightly smaller molar concentrations are γ-aminobutyric acid and glutamine which are both closely related to glutamic acid. We know little about the function

[1] The following abbreviations are used throughout this paper:
CoA: coenzyme A
DPN: diphosphopyridine nucleotide
ATP: adenosine triphosphoric acid
TOCP: tri-*ortho*-cresyl phosphate
DFP: di*iso*propylfluorophosphonate

of creatine apart from its role in phosphagen, but we know many reactions in which glutamic acid is concerned. First, it can undergo oxidative deamination, being first oxidized in presence of DPN to give iminoglutaric acid which is then deaminated to give α-ketoglutaric acid. This member of the tricarboxylic acid cycle readily forms glutamic acid in presence of ammonia and reduced DPN; this is probably an important method for removing the ammonia which is split from the amide group of proteins during brain activity (Vrba, 1955), and the vitamin nicotinic acid is essential. Secondly, glutamic acid is amidated in presence of ammonia to give glutamine, which is the main transport form of ammonia in the body and which may be concerned in the neurological manifestations of agene poisoning so well studied by Mellanby (1951); the toxic agent, methionine sulphoximine, is structurally related to glutamine and decreases the capacity of brain slices to synthesize acetylcholine, this inhibition being removed by glutamine. Thirdly, glutamic acid is readily transaminated, combining with oxaloacetate to give α-ketoglutarate and aspartic acid. Another vitamin, pyridoxal phosphate, is necessary for this reaction. Fourthly, brain contains an extremely active glutamic decarboxylase which forms γ-aminobutyric acid and also needs pyridoxal phosphate.

It is impossible to discuss here the role of ionic transport in the maintenance of neuronal structure and in activity. According to Hodgkin (1950), the immediate source of energy for the nervous impulse is the exchange of small amounts of sodium and potassium ions; and Lorente de Nó (1947) concluded that the maintenance of the resting potential depends primarily on intraneural oxidative processes. Glutamate is probably not concerned in these processes, whereas the four vitamins already mentioned (thiamine, pantothenic acid, nicotinic acid and riboflavin) certainly are. But Krebs and his colleagues (e.g. Krebs, Eggleston & Terner, 1951) have shown that glutamate is specifically required for ion transport in brain; as already mentioned, pyridoxal phosphate is intimately connected with the metabolism of glutamate.

Thiamine and pantothenic acid are needed for the oxidation of pyruvate not only in nerve cells; they are also directly concerned with the nerve fibre and in particular with the synthesis of acetylcholine which is generally regarded as essential for conduction in nerve and across certain synapses. The compound is synthesized by choline acetylase in presence of ATP (Nachmansohn & Machado, 1943), the acetyl group being transferred from acetyl-CoA in the formation of which thiamine and pantothenic acid are concerned. Acetylcholine is hydrolysed by acetylcholinesterase, and this enzyme, like thiamine, is mainly concentrated in the nerve sheath rather than in the axoplasm (Nachmansohn & Steinback, 1942; Muralt, 1943; the role of thiamine in peripheral nerve has been reviewed by Muralt, 1947).

From this brief summary of biochemical evidence that five vitamins (thiamine, pantothenic acid, nicotinic acid, riboflavin and pyridoxine) are concerned with the function of neurones, we may pass to consider briefly the effects of deficiency of each in turn.

2. Thiamine

Many experiments upon the effect of vitamin deficiencies upon the nervous system have been vitiated by failure to control various factors: the diet may have been deficient in more than one nutrient; the effects of inanition may have been overlooked; the duration and rapidity of onset of the deficiency may not have been considered; inadequate histological methods, of fixation and of staining, may have been used; species differences may have been ignored. A striking effect of deficiency of thiamine is anorexia, and starvation produces changes in the myelin sheaths of nerves.

There is no doubt that chronic deficiency of thiamine produces degenerative changes in the nervous system of pigeons (Swank, 1940; Shaw & Phillips, 1945); but various authors (e.g. Follis, 1948) have considered this as unproved for any mammal. Two years ago we obtained proof that degeneration of peripheral nerves in the rat can in fact be caused by chronic deficiency of thiamine (North, 1953; North & Sinclair, to be published); and Rodger (1953, 1954) similarly produced degenerative changes in the visual pathway. Acute deficiency in the pigeon produces a condition analogous to Wernicke's encephalopathy in man (Prados & Swank, 1942); in swine it causes myocardial necrosis and dilatation, with sudden death from heart failure brought on more rapidly by exercise (Follis, Wintrobe, Miller & Stein, 1943). The function of thiamine has been reviewed by Peters (1953) who was responsible for much of the fundamental work.

Work such as this on lower animals, together with clinical observations in man, has helped us to understand—although still imperfectly—the sequence of events in deficiency of thiamine which may be summarized somewhat as follows (see Sinclair, 1953; but contrast Platt, 1951). Thiamine, as cocarboxylase, takes part in the oxidation of α-keto acids and therefore, when it is deficient, pyruvate accumulates. This compound is toxic and may be responsible for the anorexia; pyruvic aldehyde (methyl glyoxal) also accumulates (Salem, 1954) and is more toxic (see Kun, 1950). Diets relatively high in carbohydrate but deficient in thiamine will increase the accumulation, and the diminished metabolism is likely to be particularly important in those cells that have a predominantly carbohydrate metabolism (such as nerve cells) or that use pyruvate extensively (such as cardiac muscle cells); nerves may also function imperfectly because thiamine is required for the synthesis of acetylcholine. In acute deficiency in man, either pyruvate accumulates in the lower parts of the brain and probably causes vascular dilatation and minute haemorrhages with the clinical syndrome of Wernicke's encephalopathy, or acute cardiac beriberi is produced; both respond dramatically to therapy with thiamine. Chronic mild deficiency with a diet relatively high in carbohydrate produces dry beriberi, the failure of nerve function starting distally in the longest peripheral neurones. This may occur through the toxic effect of pyruvate or possibly through the lesion in pyruvate metabolism in their cells: it is logical though not necessarily correct to believe, despite the evidence of accumulation of pyruvate and of its toxicity, that the damage is not a poisoning, and that a diet high in carbohydrate depletes the body of thiamine faster than does one high in fat or low in calories (see Gruber, 1953). In chronic deficiency there is also anorexia which is probably an effect of pyruvate upon the appetite centre in the hypothalamus. This causes inanition which is accompanied by oedema such as occurs in other cases when the diet is low in calories; in wet beriberi and in famine oedema there is adrenal hypertrophy, and it is possible that the oedema, which depends partly on the amount of salt ingested, is caused by increased retention of salt in the body, perhaps through increased secretion of aldosterone.

There are certain resemblances between deficiency of thiamine and poisoning with certain organophosphorus compounds. In both the bird is susceptible, and the rat resistant; in birds the histological lesion in peripheral nerves in either case is identical (Barnes & Denz, 1953). Though these compounds (e.g. TOCP) do not inhibit the in-vitro oxidation of pyruvate (Earl, Thompson & Webster, 1953), this is not necessarily relevant, because there is some evidence that they are changed after oral administration into the toxic compound; since it has been found that thiamine does not affect the toxicity (Davies & Fenton, personal communication), it was possible that these compounds inhibited the phosphorylation of thiamine. But, in confirmation of Earl *et al.* (1953), Mr Sacks and I have found that in the hen there is no rise in blood pyruvate during poisoning with TOCP or DFP, and administration of cocarboxylase has no effect upon the toxicity; lipoic acid is also ineffective, which is interesting since organophosphorus compounds combine with it. It has been supposed that the inhibition of pseudo-cholinesterase is not the relevant factor in neuropathy, since there is no correlation between the toxicity and degree of inhibition (Davison, 1953). But Mr Sacks has pointed out that Davison's Table III indicates that the three organophosphorus compounds that produce paralysis in hens are the only three which completely inhibit pseudo-cholinesterase in the cervical spinal cord. It has been suggested that TOCP is an antagonist of vitamin E (Bloch & Hottinger, 1943; Draper, James & Johnson, 1952).

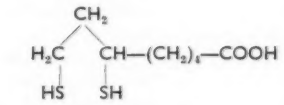
3. Pantothenic Acid

This vitamin, as part of coenzyme A, is intimately concerned with the oxidation of pyruvate, as is thiamine. One might therefore expect combined deficiency of the two vitamins to affect neurones very markedly. Unexpectedly we found that superimposing repeated acute deficiencies of thiamine upon chronic deficiency of pantothenic acid produced no demonstrable degenerative changes in the long nerves or spinal cord of the rat (North, 1953; North & Sinclair, to be published); we have therefore failed to support the hypothesis of Wintrobe, Follis, Humphreys & Stein (1944) that deficiency of pantothenic acid is responsible for the lesions in the nervous system previously ascribed to deficiency of thiamine. Deficiency of pantothenic acid produces convulsions and coma in dogs (Schaefer, McKibbin & Elvehjem, 1942), ataxia in monkeys (McCall, Waisman, Elvehjem & Jones, 1946), degenerative lesions of the spinal cord in chicks (Phillips & Engel, 1939) and degenerative changes in peripheral nerves, the posterior roots and posterior columns of the cord in swine (Swank & Adams, 1948; Follis & Wintrobe, 1945).

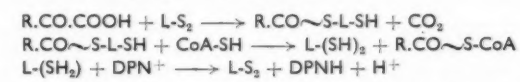
This condition in swine bears some resemblance to the "burning foot" syndrome in man which appeared during the Spanish Civil War and in the Second World War; there is reason to believe that this is in fact cured by pantothenic acid (Gopalan, 1946; Peraita, 1948). By using an analogue of pantothenic acid, Bean & Hodges (1954) have induced deficiency in four human volunteers; these became easily fatigued, quarrelsome and discontented, and paraesthesias and "burning feet" were recorded. Though some of the effects observed may have been caused by toxicity of the analogue, there is no doubt that deficiency of pantothenic acid was produced. Such deficiency is likely to be very rare in peacetime, since the vitamin is widely distributed in foods.

4. Other Hyperpyruvaemic Neuropathies

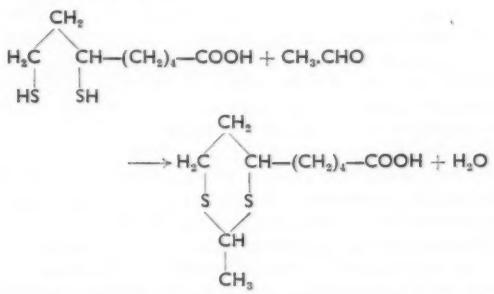
It would appear that, although thiamine and pantothenic acid are both concerned with the oxidation of pyruvate, the lesion in the nervous system is different in the two deficiencies. One would expect hyperpyruvaemia in both deficiencies and this is well known to occur in deficiency of thiamine in man and lower animals. Certain neuropathies in man are indistinguishable by the clinical signs alone from that of deficiency of thiamine; in this, marked tenderness of the calf muscles is a striking feature. Arsenical, alcoholic and one form of diabetic neuropathy are examples of these neuropathies. A very similar biochemical lesion in the nervous system could be produced by deficiency of thiamine or of pantothenic acid, or by interference with the production or catalytic function of lipoic acid:



This compound, which is 6:8-dithio-octanoic acid, can also exist in the oxidized form (—S—S—) and may actually combine with the 6-amino group of the pyrimidine ring of thiamine to give lipothiamide (see Reed, 1953). From the work of Reed, Gunsalus and others the changes can be formulated thus (where L represents the octanoic residue of lipoic acid, CoA-SH represents reduced coenzyme A, and DPNH reduced diphosphopyridine nucleotide):



Lipoic acid is presumably formed in the liver, and the reduced form combines readily with certain metals such as tervalent arsenic or bivalent mercury and with aldehydes or ketones to give a stable ring system:



We might then expect "deficiency" of lipoic acid: (i) if the liver (or other organ) failed to make sufficient—which might occur in liver disorders; (ii) if the combination of lipoic acid with some active centre was blocked either by failure of this enzymic reaction or by some compound with a similar structure (such as tetraethylthiuram disulphide (Antabuse), or dithiobiuret); (iii) if there was a wrong redox potential for the oxidation and reduction of lipoic acid, possibly in deficiency of vitamin B_{12} (see below); (iv) if the concentration was raised in the body of some substance (such as arsenic, mercury, carbon disulphide, methyl glyoxal, pyruvate, acetaldehyde or acetoacetate) which combines very readily with lipoic acid to

form a stable compound. In acute severe deficiency of lipoic acid we might expect coma or respiratory failure because the metabolism of carbohydrate in the neurones of the higher centres would be deranged; in chronic less severe deficiency we might expect peripheral neuropathy through decreased metabolism in the cell body and hence trophic disturbances starting distally in the long axons. Severe deficiency of arsenic produces death from respiratory failure; chronic use of arsenicals with one hand can produce neuropathy in the related limb alone; chronic ingestion of arsenic produces neuropathy: in all these cases the neuropathology could be caused by combination of arsenic with lipoic acid. Severe diabetic acidosis produces coma, and chronic uncompensated diabetes can produce a form of neuropathy similar to that of deficiency of thiamine: in both cases a ketone body could be inactivating lipoic acid, administration of which would be expected to reduce the acidosis without administration of insulin. Severe alcoholism causes coma or encephalopathy; chronic alcoholism, peripheral neuropathy. I have pointed out elsewhere (Sinclair, 1955) that this neuropathy could be caused by acetaldehyde inactivating lipoic acid, and that Antabuse which is used clinically because it combines with acetaldehyde also produces neuropathy. It is possible that hepatic coma could be caused by deficiency of lipoic acid and relieved by its administration.

But this is not the only possible way in which alcohol and diabetes might cause peripheral neuropathy: some years ago I found that both alcohol and arsenic interfered with the phosphorylation of thiamine in liver, and normal thiamine but low cocarboxylase levels were found in three subjects of diabetes of whom one had cardiac failure and the other two had neuropathy (Goodhart & Sinclair, 1940). Italian workers (Bisceglie, 1946; and, in particular, Siliprandi and colleagues, see Siliprandi & Siliprandi, 1951) have demonstrated a decreased phosphorylation of thiamine in diabetes. If lipoic acid were needed for this phosphorylation, the two hypotheses advanced above (namely, that inactivation of lipoic acid or failure of phosphorylation of thiamine can cause peripheral neuropathy) become unified, and Bishop Occam would be appeased.

In either case, raised pyruvate would be expected, and this has not always been found. It occurs in arsenical poisoning (Peters, Sinclair & Thompson, 1946) and has sometimes (Markees & Meyer, 1949) but not always (Bueding, Wortis, Fein & Esturonne, 1942; Martin, 1953; Smith & Taylor, 1955) been alleged to occur in diabetic neuritis; here the value of the customary oral dose of glucose is negligible and blood pyruvate following intravenous injection of it must be studied. The fact that blood pyruvate is alleged to be normal in diabetic coma (Van Marken Lichtenbelt & Florijn, 1952) does not necessarily invalidate the use of lipoic acid in this condition, since the inactivation of this coenzyme by a ketone body could be sufficiently acute to cause coma without a marked rise in pyruvate. It is perhaps relevant to add that an extract of pregnant mammalian liver has been alleged to be efficacious in diabetic neuropathy (Rabinowitch, 1952); it is possible that this might contain lipoic acid and indeed, if the requirement of lipoic acid is increased in pregnancy, this coenzyme might be brought into the picture of gestational polyneuropathy.

A form of peripheral neuropathy of great interest is that accompanying bronchial carcinoma. That malignant disease may be associated with neuropathy has been known for years, but this particular type of malignancy is especially prone to cause it. Denny-Brown (1948) thought that the carcinoma might produce a product interfering with the metabolism of pantothenic acid; and the lesions in the nervous system, as in the case described by McCaughey & Millar (1955), can be very similar to those of deficiency of pantothenic acid in swine. Apparently blood pyruvate may be raised, but is not necessarily high (Heathfield, 1952; Heathfield & Williams, 1954; Kremer & Pratt, 1952; Henson, Russell & Wilkinson, 1954); some evidence for the production of a toxic substance by the tumour has been produced (Semple & McCluskie, 1955). Malignant tissue glycolyses aerobically, unlike normal nucleated tissues, and produces lactate and pyruvate; the pyruvate or some other metabolite may combine with lipoic acid, thereby producing a partial deficiency of this coenzyme and so a block in the metabolism of carbohydrate in neurones. This seems no worse than any other speculation concerning the association of neuropathy with malignant disease, particularly of the lung; the only case we have so far examined (carcinoma of bronchus with polyneuropathy) had an abnormal concentration in blood of *p*-hydroxyphenylpyruvic acid.

5. Vitamin B_{12}

We may briefly consider this vitamin next, partly because peripheral neuropathy is commonly found in association with subacute combined degeneration of the cord, and partly because it may be concerned with the metabolism of pyruvate or a derivative, and with lipoic acid. Vitamin B_{12} has been alleged to be useful in relieving the pain in four cases of nutritional neuropathy (Bean, Franklin & Sahs, 1950) and in treating diabetic neuropathy (Sauer & Dussler, 1954; but contrast Shuman & Gilpin, 1954). The biochemical role of vitamin B_{12} is reviewed more fully by Lester Smith on p. 52 of this number of the Bulletin.

In pernicious anaemia an alteration in metabolism of tyrosine and its derivatives has been described (Swendseid, Wandruss & Bethell, 1947; Abbott & James, 1950); there is also an increase in the amount of *p*-hydroxyphenylpyruvic acid in blood (Bussi, Pozza, Eridani, Fava & De Micheli, 1953). We believe that this compound (which combines with lipoic acid) or a derivative of it might be toxic to the neurones of the spinal cord. One might in fact suppose that pyruvate was toxic particularly to peripheral neurones (see above), *p*-hydroxyphenylpyruvate to the neurones of the cord, and phenylpyruvate to those of the brain: for various workers (e.g. Udenfriend & Bessman, 1953; Armstrong & Tyler, 1955; Woolf, Griffiths & Moncrieff, 1955) have shown that in phenylketonuria there is a metabolic error in the conversion of phenylalanine to tyrosine with consequent accumulation of derivatives of the former, one or more of which appears to be responsible for the mental deficiency. The toxic compound in each case might not be pyruvate (or the phenyl or phenolic derivative) since, for instance, in scurvy *p*-hydroxyphenylpyruvate is excreted in increased amounts but there are no characteristic neurological signs. With this hypothesis in mind we investigated, with the collaboration of Dr F. Wokes and Dr J. Badenoch, a group of persons (vegans) who subsist upon diets almost devoid of vitamin B_{12} and who tend to develop neuropathy. We found that in general there appears to be increased *p*-hydroxyphenylpyruvate in the blood of those with lowered blood vitamin B_{12} (Wokes, Badenoch & Sinclair, 1955). This substance, or a derivative of it, might be directly

toxic to neurones or might combine with lipoic acid. In the latter case raised blood pyruvate might be expected, but we have not found this in our patients, although Earl, El Hawary, Thompson & Webster (1953) have done so in cases of subacute combined degeneration of the cord. There might be a more direct connexion between vitamin B_{12} and lipoic acid: according to Register (1954) there is a decrease in non-protein sulphydryl groups such as reduced glutathione in B_{12} deficiency; Ling & Chow (1954a, 1954b) have also found a decrease in reduced glutathione and have claimed impaired carbohydrate metabolism (reduced glutathione is a coenzyme for the oxidation of methyl glyoxal which is toxic and readily combines with lipoic acid); and from the preliminary work of Dubnoff & Bartron (1954) and others it appears that vitamin B_{12} keeps the non-protein disulphide groups reduced and interacts with coenzyme A. This may be the basis for its connexion with the formation of ribose and hence of ribonucleic acid, deficient formation of which in spinal motor root cells of the guinea-pig appears to occur after muscular fatigue in deficiency of vitamin B_{12} (Gomirato, 1954). It is obvious that much more experimental work needs to be done before we can understand the role of vitamin B_{12} in the nervous system. But, if it is directly concerned with maintaining the correct redox potential for the sulphydryl groups of lipoic acid, this cannot be a general biological reaction since higher plants need reduced lipoic acid for photosynthesis (see Calvin, 1955) and do not contain vitamin B_{12}. In them, however, reduction of lipoic acid might be assisted by ascorbic acid and this might be the main function in higher plants of the high quantities of ascorbic acid which can readily form an additional compound with lipoic acid.

6. Pyridoxine

The connexion of this vitamin, B_6, with the metabolism of glutamic acid in brain was mentioned above. Deficiency in lower animals produces convulsions which can be relieved by glutamic acid; certain convulsions in children are cured by pyridoxine (Molony & Parmelee, 1954; Coursin, 1954; Hunt, Stokes, McCrory & Stroud, 1954). By the use of an antagonist, deoxypyridoxine, peripheral neuropathy has been produced in man and cured with pyridoxine (Vilter, Mueller, Glazer, Jarrold, Abraham, Thompson & Hawkins, 1953); peripheral neuropathy also arises as a complication of therapy with isonicotinic acid hydrazide (isoniazid), since this readily forms with pyridoxal a hydrazone which is excreted in the urine.

There is another way in which deficiency of vitamin B_6 may affect the nervous system. According to Woolley & Shaw (1954), 5-hydroxytryptamine ("serotonin") is an essential hormone for the nervous system. Pyridoxal phosphate is required for the formation of this by decarboxylation of 5-hydroxytryptophan, and in deficiency of the vitamin the concentration of 5-hydroxytryptamine is decreased. This might be the cause of convulsions.

7. Nicotinic Acid, Riboflavin and Other Members of the Vitamin B Complex

Nicotinic acid and riboflavin have already been mentioned as being essential for enzymic reactions in brain metabolism. From the work of Sydenstricker (1943), Jolliffe (1941) and others, deficiency of nicotinic acid is known to produce encephalopathy and mental deterioration; occasional cases are to be found in mental hospitals. Although deficiency of riboflavin causes neuropathy in lower animals, there is no proof that it does so in man.

Biotin deficiency causes spasticity in rats and is alleged to cause mental depression in man (Sydenstricker, Singal, Briggs, DeVaughn & Isbell, 1942). Both inositol and choline occur in phospholipids; the latter is a source of labile methyl groups and a constituent of acetylcholine.

* * *

Sir Edward Mellanby correctly pointed out twenty-one years ago that the nervous system came within the pale of nutrition. The facts and speculations briefly included in this article support this view, and although much experimental work has been done since Mellanby stated it, a great deal more is still required to explain the function of vitamins in the nervous system and neurological manifestations of their deficiencies.

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VITAMINS AND THE SKIN

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1 Biochemistry of the skin
2 Fat-soluble vitamins
3 Pyridoxine
4 Riboflavin
5 Nicotinic acid
6 Other members of the vitamin B complex
7 Ascorbic acid
References

In 1777 Milly took a warm bath; small bubbles were noticed attached to the surface of the body, and these were collected and shown by Lavoisier to be carbon dioxide (Milly, 1777). Though it was objected that the water rather than Milly might have contributed the gas, the absorption of oxygen and discharge of carbon dioxide by the skin was soon proved by a number of observers, including Lavoisier. Despite the important functions of the skin in protection, in temperature and water regulation, in secretion and absorption, and despite the ease with which it can be observed, surprisingly little is known about its metabolism.

1. Biochemistry of the Skin

As indicated in my preceding paper in this number of the Bulletin, "Vitamins and the nervous system", we have detailed knowledge of the biochemistry of the nervous system, and in the light of this we can begin to interpret disorders of this system; but we do not even know for certain if the citric acid cycle functions in skin. It has been claimed that it does not: Barron, Meyer & Miller (1948) obtained no evidence that citrate and α-ketoglutarate were used by skin and they found no *iso*citrate dehydrogenase; they, like others, found that fat as well as carbohydrate was oxidized, as indicated by the R.Q. of 0.85 and by the oxidation of butyrate and acetoacetate, and that ammonia was formed from various amino acids. The evidence against the citric acid cycle in skin is negative evidence and not conclusive: Burton & Williams (1950) found no tyrosinase in human epidermis, but this enzyme is present in an inhibited form (Fitzpatrick, Becker, Lerner & Montgomery, 1950). It is reasonable to conclude that energy metabolism in the skin occurs as in most other tissues through glycolysis, the citric acid cycle and the cytochrome system; therefore the vitamins nicotinic acid, riboflavin, thiamine and pantothenic acid are required, and lipoic acid is also involved. If allowance is made for inactive material, the respiration of the cells of epidermis is very rapid.

The extensive work upon the metabolism of protein and amino acids in the skin cannot be reviewed here. Pyridoxine may be involved in this important turnover: as much as 40% of the daily intake of protein may be lost by desquamation (Peters, 1945). Disorders of pigmentation of skin and hair are common in nutritional disturbances, and the fundamental process is now well understood (Lerner & Fitzpatrick, 1950): tyrosinase, which is activated by copper, is inhibited by thiol groups which bind copper, and, when these groups are oxidized to disulphide groups (as by ultra-violet light), tyrosinase acts in the production of melanin. These groups are of fundamental importance in keratinization as well as in pigmentation, and it is not surprising that arsenic, which readily combines with them, increases both (Peters, Sinclair & Thompson, 1946).

2. Fat-Soluble Vitamins

The basal cells of the epidermis divide and form prickle cells which, however, are themselves capable of dividing. As the cells move towards the surface, keratinization occurs, starting when the cells are in the upper Malpighian layer. Vitamin A, which has not been demonstrated in the skin and possibly occurs in a combined form, decreases keratinization. The remarkable effects of excess of vitamin A upon the skin, so well studied by Dr Honor Fell and Sir Edward Mellanby, are described on p. 35 of this issue of the Bulletin (Fell, 1956).

Increased keratinization, like increased pigmentation, is a general reaction to injury, and each process is catalysed by copper. In the former, copper catalyses the conversion of the thiol groups of cysteine into the disulphide bridges found in keratin; although there is no doubt that this occurs during keratinization in hair, it is not so certain for epidermis (Rudall, 1952). The decrease in thiol groups which accompanies increased keratinization might release tyrosinase from inhibition and be the mechanism of the alleged pigmentation of the skin in deficiency of vitamin A (Mu, Frazier & Pillat, 1937). Fischer (1924) believed that keratinization was a response to poor nutrition, but this is not necessarily so. It was observed as a normal event in epithelial cultures from chick embryos by Strangeways & Fell (1926) and by Fell (1929); no increased keratinization was found by Miszurski (1937) when he decreased the vitality of these cultures by various methods.

The pathogenesis of the epithelial lesions in deficiency of vitamin A has been characterized by Wolbach, who was responsible for much of the early work, as "atrophy of the epithelium concerned, reparative proliferation of basal cells, and growth and differentiation of the new products into a stratified keratinizing epithelium" (Wolbach & Bessey, 1942). This has been well studied in epithelial structures other than the skin; but, as Sullivan & Evans (1945) point out, it is difficult to visualize atrophy followed by metaplastic epithelial hyperkeratinization in the skin which is already keratinized. They obtained no lesions in the skin of rats fed a diet deficient in vitamin A unless atrophy was first induced by deficiency of members of the B complex. Numerous claims have been made that various forms of follicular hyperkeratosis in man are caused by deficiency of vitamin A (for literature, see Ramalingaswami & Sinclair, 1953). Unfortunately, the therapy used has not been confined to vitamin A alone, since the vitamin has been administered as a concentrate which contains essential fatty acids. We therefore studied in considerable detail acute, chronic and partial deficiency of vitamin A in the rat, about 100 animals being examined. There was complete disappearance of interfollicular and subcutaneous fat, with marked reduction in thickness of the skin. There was some

atrophy of the basal and Malpighian layers of the epidermis, and loose hyperkeratosis of the stratum corneum. The upper third of the hair follicles was dilated and hyperkeratotic, with atrophy of the lining epithelium throughout the follicle; there was atrophy of the sebaceous glands. These changes were consistent and unlike those found in phrynoderma in man.

Little is known about the reason for the atrophy of the transitional layers and hyperkeratosis in deficiency of vitamin A: apart from its well-characterized relation to the cycle in the rods, we know almost nothing about the biochemical role of the vitamin.[1] It is tempting to suppose that the double bonds in the aliphatic side-chain are concerned with the oxidation and reduction of thiol groups. Morton has suggested that it may be concerned in preventing the dehydrogenation of sterols to β-naphthalenic compounds (Lowe, Morton & Harrison, 1953); in this connexion it is of interest that naphthalene derivatives produce acne in man and the hyperkeratosis known as "X disease" in cattle[2], the latter being prevented, at least in part, by vitamin A (Hoekstra, Hall & Phillips, 1954).

Hyperkeratosis also occurs in deficiency of essential fatty acids (EFA), first recognized by Burr & Burr (1929) in the rat. These have now been shown to be essential for several other species: the flour-moth (Fraenkel & Blewett, 1947), chicken (Reiser, 1950), dog (Hansen & Wiese, 1951), hamster (Christensen & Dam, 1952), calf (Lambert, Jacobson, Allen & Zaletel, 1954), lamb and goat (Cunningham & Loosli, 1954), guinea-pig (Reid, 1954), and probably for the pig (Witz & Beeson, 1951); the evidence for their vitamin-like character in man has been discussed elsewhere (Sinclair, 1952).

Since the evidence for vitamin A's being concerned in the production of phrynoderma in man was unsatisfactory, and deficiency of EFA was a possible cause, we studied carefully the changes in the skin of the rat in deficiency of EFA and found them to be very different from those caused by deficiency of vitamin A (Ramalingaswami & Sinclair, 1953). There is surprisingly little reduction in subcutaneous fat and a marked increase in the thickness of the epidermis (in the strata Malpighii, granulosum and corneum); the sebaceous glands are initially hypertrophic; the skin is very sensitive to injury. A very interesting change in the skin is its increased permeability to water in EFA deficiency (Sinclair, 1952; Basnayake & Sinclair, 1954); this can be demonstrated in a variety of ways and explains, at least in part, the increased consumption of calories and water in animals deficient in EFA. We believe that EFA may be required for the formation of cell membranes or of the cytoskeleton; therefore, in deficiency, growth ceases and the prickle cells with their tonofibrils presenting relatively enormous cell membranes are affected, and acanthosis occurs; the transitional layers of the skin maintain its partial impermeability to water, and the increased loss of water through the skin exacerbates the dermatosis (Basnayake & Sinclair, 1956).

3. Pyridoxine

Superficially, the lesion in the skin of the rat in deficiency of EFA resembles that found in deficiency of pyridoxine (Sullivan & Nicholls, 1940; Antopol & Unna, 1942), but in fact the two conditions are quite distinct (Ramalingaswami & Sinclair, 1953). Essentially, deficiency of pyridoxine causes atrophy of the epidermis, the hair follicles and the sebaceous glands, with loose hyperkeratosis and very marked loss of subcutaneous fat. In more advanced lesions found on the paws, there is vascular engorgement of the corium, oedema, marked acanthosis and hyperkeratosis, and the permeability to water is increased (Basnayake & Sinclair, 1956); the lesion is then closer to that found in deficiency of EFA, which it also resembles in great sensitivity to injury—as caused by ultraviolet light. But whereas deficiency of EFA probably affects primarily the prickle cells, deficiency of pyridoxine may produce its effects primarily through the vascular system. There is increased vascularity visible in the skin before any morphological change occurs in the epidermis; it is possible that this increased vascularity is secondary to a rise in body-temperature caused by uncoupling of oxidative phosphorylation, which could cause the wastage of calories so characteristic of pyridoxine deficiency and the creatinuria (Dinning, 1955), since creatine seems to be excreted in the urine whenever uncoupling of oxidative phosphorylation occurs (for instance, after dinitrophenol or thyroid administration, or in deficiency of vitamin E). These possibilities we are investigating. Although at one time we thought the skin signs might be related to the amount of histamine in the skin, we now think this improbable, since in deficiency of pyridoxine the amount of histamine in skin is significantly diminished, but lowering it still more by simultaneous administration of the powerful histamine-liberator, "substance 48/80", does not affect the lesions (Sinclair, Mayer & Buxton, 1955).

The important work of Vilter and his colleagues has demonstrated strikingly the effect of deficiency of pyridoxine in man as revealed by administration of deoxypyridoxine (Vilter, Mueller, Glazer, Jarrold, Abraham, Thompson & Hawkins, 1953). Further work is required to elucidate the role of pyridoxine in therapy of seborrhoeic dermatitis in man.

4. Riboflavin

Deficiency has been extensively studied in the rat (see, for example, Sullivan & Nicholls, 1941); changes have also been described in the mouse (Lippincott & Morris, 1942; Fenton & Cowgill, 1947), monkey (Cooperman, Waisman, McCall & Elvehjem, 1945), calf (Wiese, Johnson, Mitchell & Nevens, 1947), pig (Wintrobe, Buschke, Follis & Humphreys, 1944) and other animals. In general there is atrophy of the epidermis, and hypertrophy followed by atrophy of the sebaceous glands.

In man, Sebrell & Butler (1939) produced cheilosis, angular stomatitis and dermatitis of the nasolabial folds; scrotal or vulval dermatitis also occurs (Hills, Liebert, Steinberg & Horwitt, 1951).

5. Nicotinic Acid

As with riboflavin, there is little recent work that has added to our understanding of the skin lesions that occur in deficiency of nicotinic acid, the classical manifestation of which is pellagra in man; the lesions in kwashiorkor have recently been admirably summarized (Trowell, Davies & Dean, 1954).

In deficiency of nicotinic acid, the initial changes in the skin appear to be rarefaction of the superficial part of the corium and dilatation of the blood vessels (Denton, 1925, 1928; Moore, Spies & Cooper, 1942). In the epidermis there is disturbed keratinization, and the epidermis separates from the corium. Any form of trauma greatly exacerbates the lesions.

[1] See also papers by Morton & Goodwin (p. 37) and by Pirie (p. 32) in this number of the Bulletin.—ED.

[2] See Coates, Kon & Porter, p. 61 of this number of the Bulletin.—ED.

6. Other Members of the Vitamin B Complex

Deficiency of pantothenic acid very quickly produces lesions in skin in the cotton rat (Elvehjem, McIntyre & Schweigert, 1944) and in pigs (Wintrobe, Follis, Alcayaga, Paulson & Humphreys, 1943). The lesions were carefully studied in the rat by Sullivan & Nicholls (1942) who found hyperkeratosis and acanthosis followed by atrophy of the epidermis. Copper appears to be poorly utilized and so accumulates, which may account for the greying of the hair (Hundley & Ing, 1951).

The skin has been carefully studied in deficiency of biotin in the rat (Sullivan & Nicholls, 1942) and mouse (Montagna, 1950). Deficiency of inositol causes alopecia in the mouse (Woolley, 1940).

Estimations of various vitamins of the B complex in human skin have been made by Lee, Lerner & Halberg (1953).

7. Ascorbic Acid

A fairly exhaustive recent review of this vitamin (Lloyd & Sinclair, 1953) and a masterly account of its importance for intercellular substances (Wolbach, 1953) make unnecessary any further attempt to correlate the biochemical function of the vitamin with the characteristic perifollicular haemorrhage and the follicular hyperkeratosis that occur in scurvy. The haemorrhages are caused by weakening of the collagen supporting the capillary wall rather than by any deficiency in the wall itself (Lee & Lee, 1947).

* * *

Dante in *Il Purgatorio* described a troupe of souls who were fasting; their pale dry parched skin was blotched pigmented and desquamating. Hyperkeratosis is the commonest manifestation of nutritional deficiency and has often been discussed (McCance & Barrett, 1951; Platt, 1953; Sinclair, 1948). Follicular hyperkeratosis can undoubtedly be caused by deficiency of aliments or of ascorbic acid or of essential fatty acids (in lower animals at least), but it also occurs particularly in rather obese girls at the age of puberty (though without dryness of the skin), and it may occur in deficiency of vitamin A. The common factor may be faulty metabolism in the epidermal cells, produced by lack of substrates or of catalysts or (as in the obese) by vascular changes. Not until we know more about the respiration of the skin, observed by Milly when lying in a bath two centuries ago, shall we be able to interpret the changes in it that are produced by deficiencies of vitamins.

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RECENT WORK ON VITAMINS: BONES

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Research work on vitamins and bone is so intimately associated with the name of Sir Edward Mellanby that it is difficult to think of one without the other. Before he died Sir Edward had promised to write this article for the Bulletin. Unfortunately, he was not given the time, and we have been quite unable to add those personal touches and opinions which would have contributed so much to the article. We have done our best, but we cannot pretend to be masters of the subject, as he was.

A number of vitamins are now well known to play some part in developing the orderly structure of bone. Gross skeletal changes are produced by deficiencies of vitamins A, C and D, but a sufficiency of certain B vitamins is also necessary for the perfect formation of the skeleton. The main effects of all vitamin deficiencies on the skeleton are produced during the period of active growth when the bones are enlarging rapidly.

1. Vitamin A

a. Effects of Deficiency

Mellanby was a shrewd observer, and in his early work on the experimental production of rickets in puppies he noticed that animals that were being fed on diets containing no cod-liver oil sometimes showed nervous symptoms, including incoordination of movement. In later years he traced this effect to a deficiency of vitamin A, and showed that degenerative changes could be demonstrated in many parts of the central and peripheral nervous systems of the affected animals. Finally, Mellanby (1938, 1941, 1947) presented evidence that the nervous disorders resulted from pressure on the neural tissue owing to overgrowth of bone in certain parts of the cranium and vertebrae, notably around the foramina through which the nerves emerged from the cranium. He gave a delightful account of all this work in its historical sequence in the Abraham Flexner Lectures (Mellanby, 1950).

From Mellanby's work and that of other investigators it is now known that nervous disorders can be produced in rats, ferrets, dogs, pigs, sheep, calves, rabbits, chickens and ducks, if the diet contains too little vitamin A during the early stages of growth. The primary cause, however, is still uncertain. In their studies on calves, for instance, Moore, Huffman & Duncan (1935) and Moore (1939) found evidence of narrowing of the optic foramen and atrophy of the optic nerve at the point where it passes through the foramen, and concluded that constriction of the nerve by bony pressure was the cause of the blindness which developed. They suggested that the bone deformity could have arisen through an increase in intracranial pressure during early growth. Phillips & Bohstedt (1938), however, failed to find stenosis of the optic foramen in rabbits which had been fed on an equally deficient diet and had become ataxic and blind. Moore & Sykes (1940) showed that a rise in cerebrospinal fluid pressure accompanied the nervous disorders which preceded blindness in calves, and Mellanby (1941) also found a rise in cerebrospinal fluid pressure in puppies showing nervous disorders, and some indications of a general rise in intracranial pressure. In recent years much further evidence has accumulated of increased cerebrospinal fluid pressure in calves and young pigs deficient in vitamin A.

The Cambridge workers, Millen, Woollam & Lamming (1953), observed nervous disorders in young rabbits whose mothers had eaten a diet deficient in vitamin A during pregnancy and for 14 weeks before mating. Hydrocephalus was found in the young animals and was considered to be responsible for the nervous symptoms. The cause of the hydrocephalus was at first thought to be stenosis of the cerebral aqueduct, but from further studies on foetal and still-born rabbits (Millen, Woollam & Lamming, 1954) it was concluded that the primary cause was over-production of cerebrospinal fluid.

From extensive histological studies, mainly on rats, of endochondral bone growth as well as the nerve lesions produced in vitamin A deficiency, the American pathologist, Wolbach, reached the conclusion (see, for example, Wolbach & Bessey, 1941, 1942) that vitamin A deficiency causes a retardation of bone growth, especially endochondral bone formation, and that the nerve lesions result from compression of the nervous system through failure of the skeleton to enlarge sufficiently to accommodate the nervous system, which continues to grow at a normal rate. Mellanby (1947), however, considered that the explanation was not so simple and that the deposition of bone was not stopped in vitamin A deficiency, although its site might be altered. His view was that vitamin A governs the shape and texture of certain bones by controlling the position and activity of osteoclasts and osteoblasts and that, in deficiency of vitamin A, bone growth lacks the co-ordination between deposition and resorption which is responsible for normal modelling. No changes in bone structure have so far been attributed to vitamin A deficiency in man.

b. Effects of Excessive Intake

The fact that the ingestion of polar-bear liver may cause serious and acute illness has long been recognized by Eskimos and by Arctic explorers. It had also been known for many years that an excessive intake of vitamin A can have toxic effects on laboratory animals. It was not until 1943, however, that Rodahl & Moore first showed that the toxicity of polar-bear liver is due to the extremely high concentration of vitamin A which it contains.

Much of the earlier work on experimental animals was done with impure vitamin preparations and there was some doubt as to whether all the toxic manifestations were due to vitamin A alone. Moore & Wang (1943, 1945), however, showed conclusively that pure vitamin A acetate is toxic when given in massive doses to rats, and they confirmed the earlier findings of bone fragility and spontaneous fractures. Bone changes resulting from prolonged administration of excessive amounts of the vitamin have subsequently been described in a number of different species, including rats, dogs, guinea-pigs, chickens and ducks. Congenital malformations, including gross anomalies in the development of the skull, have also been described by Cohlan (1953) in young rats whose mothers had received excessive amounts of vitamin A during pregnancy. From extensive histological studies, Wolbach concluded (see, for example, Maddock, Wolbach & Maddock, 1949; Wolbach & Hegsted, 1952) that the general effect of excess of vitamin A on the skeleton is to cause acceleration of those processes that are retarded by a deficiency of the vitamin, namely, growth and maturation of epiphyseal cartilage cells, and the remodelling sequences.

In recent years a number of cases of vitamin A poisoning in man have been described in the USA. The clinical findings, which have been summarized by Gribetz, Silverman & Sobel (1951) and Knudson & Rothman (1953), include, in the case of infants, painful swellings over the long bones. *x*-Ray examination has revealed periosteal thickening in the mid-shaft region, but without histological evidence it is impossible to say how closely the bony changes which have been described in man resemble those observed in experimental animals.

c. Mode of Action

In recent years much has been learned about the biochemical mechanism responsible for the interconversion of vitamin A and its aldehyde, retinene, in the visual cycle. It has been shown that diphosphopyridine nucleotide is involved, and this knowledge may sooner or later provide a clue to the action of the vitamin in other tissues, including bone.

That excess of vitamin A can have a direct local action on bone has been shown by Barnicot (1950) in bone fragments implanted in the cerebral hemispheres of mice, and Fell & Mellanby (1950, 1952) demonstrated both disintegration of cartilage matrix and resorption of bone in foetal mouse bones grown in tissue culture. This work, which is of considerable interest, is fully described in another article in the present volume.[1]

2. Vitamin B Deficiency

Although it had previously been shown that endochondral bone growth is retarded when young animals are deprived of riboflavin, pantothenic acid or pyridoxine, only comparatively recently have detailed histological studies been made of the bone changes which take place. Levy & Silberberg (1946a, 1946b) studied the epiphyseal changes resulting from riboflavin and pantothenic acid deficiencies in mice, and concluded that the effects of the two deficiencies are not identical and differ in each case from the effects produced by reduced food intake. In the rat the bone changes in chronic riboflavin deficiency were found by Nelson, Sulon, Becks & Evans (1947) to be similar to, but more severe than, those described by Levy & Silberberg (1946a). Wolbach (1954), however, concluded that the changes in riboflavin deficiency are not specific, and both Silberberg & Levy (1948) and Wolbach (1954) have questioned whether the retardation of endochondral bone growth, which has been met with in pyridoxine deficiency, has distinct and characteristic features.

Congenital skeletal malformations were observed by Warkany & Schraffenberger (1943) in young rats whose mothers had received a diet deficient in riboflavin. The main osseous defects were shortness of the mandible and certain other bones, and fusion of the ribs, sternal centres of ossification, fingers and toes. Cleft palate was also observed in some cases.

3. Vitamin C

a. Effects of Deficiency

The basic difference between the bony changes in scurvy and in rickets is that the former arise through a defect in the formation of the matrix (osteoid), while the latter result from a failure in its calcification.

In scurvy there is no failure in the proliferation of epiphyseal cartilage cells or in their provisional calcification, but the matrix produced does not seem to gel. This abnormal matrix does not ossify, and gives rise to irregular masses of calcified cartilage in fibrous tissue (the so-called " Trümmerfeld " zone of the early German workers). On the side of this zone facing the shaft the marrow space fills with loose connective tissue (the " Gerüstmark ") and the shaft becomes rarefied. For full details of the gross and histological findings see Dalldorf (1938), Wolbach & Bessey (1942) or Follis (1948).

b. Influence on Enzyme Activity of Bone

That the phosphatase activity of bone is reduced in scurvy was shown by Gould & Shwachman (1942) and shortly afterwards by Bourne (1943). The fact that both the bone and serum phosphatase concentrations fell when vitamin C was deficient, and rose when the vitamin was administered, led Gould and Shwachman further to suggest that osteoblasts are the source of the serum enzyme. Recent work (Perkins & Zilva, 1950) from the Lister Institute of Preventive Medicine, London, has, however, shown that the serum phosphatase activity in guinea-pigs falls sooner in deficiency of vitamin C, and returns to normal later in recovery, than does the bone phosphatase concentration. From this and other evidence Perkins and Zilva concluded that the early fall in the serum level is not due to a fall in the production of the enzyme, but to a redistribution between the serum and other tissues to fulfil some protective function. They agreed that the fall in bone phosphatase may be due to a disturbance of osteoblast function caused by the scorbutic lesions. They showed too that ascorbic acid does not have an immediate effect on the phosphatase activity of the tissues, for there was no increase in the bone or serum phosphatase in scorbutic animals 18 hours after the administration of sufficient ascorbic acid to saturate the tissues, and normal levels were not regained for another two to four days.

c. Mode of Action

Vitamin C is required for the production of intercellular matrices in connective tissue as well as in bone, and present knowledge about its mode of action in bone has been derived in part from studies of other tissues, particularly the scar tissue of healing wounds. The evidence as a whole indicates that the bone defect in scurvy lies in the inability of the matrix to produce normal collagen fibres, and it seems likely that the defective constituent of the matrix is a mucopolysaccharide.

[1] See Fell, p. 35 of this number of the Bulletin.—Ed.

The exact biochemical role of vitamin C in the maturation of collagen fibres and in the organization of the interfibrillar substance is, however, not yet clear.

Biochemical studies on collagen distribution in the tissues of guinea-pigs have shown that the concentrations in certain soft tissues are not reduced in scurvy. A fall in bone collagen in scurvy was observed by Lightfoot & Coolidge (1948) and regarded as consistent with an interruption in the conversion of some precursor to collagen. A slight fall in collagen in teeth and costochondral junctions was attributed by Robertson (1950) to dilution by growth and not to destruction of collagen. Robertson concluded that, while all available evidence indicated that vitamin C was needed for collagen formation, the biochemical evidence did not support the older view, based on histological studies (see, for example, Wolbach & Howe, 1926), that vitamin C was also needed for the preservation of preformed collagen. The rate of turnover of collagen in the bones of rats is normally very slow, decreasing with the age of the animal (Perrone & Slack, 1951) and, in experiments with glycine containing ^{15}N, Robertson (1952) found that vitamin C deficiency had no specific effect on the incorporation of ^{15}N into the collagen of bone or other tissues. This and other evidence prompted Robertson to suggest that the turnover of nitrogen in collagen takes place without appreciable breakdown and re-synthesis of the collagen macromolecule.

Histological studies on healing wounds have indicated that mucopolysaccharides in the interfibrillar substance are affected in scurvy (see, for example, Penney & Balfour, 1949). In studies on bone, Sadhu (1952) found a fall in the concentration of chondroitin sulphate in the long bones of scorbutic guinea-pigs, but not in cranial bone, and Reddi & Nörstrom (1954) a reduction in the amount of ^{35}S incorporated into the chondroitin sulphate of costal cartilage.

4. Vitamin D

a. Effects of Deficiency

For centuries the disease produced in infants and children by a deficiency of vitamin D has been known as rickets. In later years the term osteomalacia was applied to the corresponding disease in adults, and in recent times a number of bone diseases have been described which, although distinct from classical rickets, appear to be related to it, and an extensive and in some cases rather confusing terminology has arisen. While classical rickets will primarily be discussed here, closely related syndromes which are of interest in trying to elucidate the mode of action of the vitamin will also be mentioned.

The discovery by Mellanby (1918) that rickets could be induced and controlled in dogs by means of diet represented the first important advance in the experimental study of the disease, and it paved the way for all the therapeutic work which subsequently revolutionized urban life in Britain. At first the theoretical aspect of this work was complicated by the association of vitamins A and D in cod-liver oil, but, once vitamin D had been separated and synthesized from ergosterol, knowledge of its effects rapidly increased. Mellanby was concerned for many years over the mysterious anti-calcifying effects of cereals, which were shown later (Harrison & Mellanby, 1939; McCance & Widdowson, 1942) to be due to phytic acid, a substance which can, in the absence of ample supplies of calcium and vitamin D, depress the absorption of calcium by combining with it and rendering it unavailable. Mellanby returned to this problem again in 1949 and studied the interactions of vitamin D, calcium and inorganic and phytic acid phosphorus in the intestinal tract, and his results were incorporated in his Flexner Lectures (Mellanby, 1950).

The discovery that rickets could be cured by vitamin D led to the recognition of the familial disease which has been termed resistant rickets, and which has been studied on both sides of the Atlantic (see, for example, Mackay & May, 1945). It remains a problem in therapeutics, for it is sometimes almost impossible to correct the bony deficiencies and maintain skeletal growth without giving toxic doses of vitamin D. A case of acquired resistance to vitamin D has also been recorded (McCance, 1947).

The bone changes in rickets have been well studied and may briefly be described as a failure of the matrix to calcify. In the long bones of children there is failure both of provisional calcification and of ossification. The cartilage and matrix continue to grow, however, thus giving rise to the enlarged "rachitic metaphysis", in which penetration by capillaries takes place only in a very irregular manner. Both in rickets and in osteomalacia, osteoid tissue is found along the trabeculae and cortex of the shaft, and there is some osteoporosis. For a detailed description of the gross pathology and histology of rickets, see Wolbach & Bessey (1942) and Follis (1948).

b. Effects of Excessive Intake

Both in man and in experimental animals the administration of excessive amounts of vitamin D concentrates leads to mobilization of minerals from the skeleton, and to metastatic deposits of calcium in many soft tissues, calcification in the kidneys being the dominant toxic effect. The onset and severity of the symptoms, however, have been found to be influenced by a variety of factors, which include not only the amount and nature of the vitamin D preparation, and the age and species of animal, but also the dietary levels of calcium, phosphorus and vitamin A, and various pathological conditions of the kidneys, endocrine system and gastrointestinal tract. From histological studies of endochondral bone growth in animals, Wolbach & Bessey (1942) concluded that moderately excessive doses accelerate provisional calcification, but that larger doses retard growth, presumably because of interference with cartilage growth.

Many cases of hypervitaminosis D in man have been described, mostly in individuals given massive doses of vitamin D concentrates in the treatment of rheumatoid arthritis or tuberculosis, and much of the clinical literature has been summarized by Wolbach & Bessey (1942), Mulligan (1947) and Chaplin, Clark & Ropes (1951). Diffuse demineralization of bones is observed in advanced cases. A disease which has come very much to the fore in British literature on paediatrics within the last few years may be due to an abnormal sensitivity to vitamin D. The infants have hypercalcaemia, a normal or high serum phosphate, and a phosphatase on the low side of normal. They lose their appetite, absorb calcium very readily and get metastatic calcification of the kidneys. Rarefaction of the bones has not been described (see Lightwood, 1952; Lightwood & Stapleton, 1953; McCance, 1953; Dawson, Craig & Perera, 1954).

c. Influence on Bone Enzymes

The presence of alkaline phosphatase in ossifying cartilage was first shown by Robison (1923), who suggested in this and later publications that phosphatase may play an important part in the calcification process by acting on a phosphoric ester to raise the local concentration of inorganic phosphate.

He did not, however, demonstrate a satisfactory substrate for the enzyme in plasma, bone or cartilage. Since that time a connexion between phosphatase and calcification has been generally admitted and as frequently discussed, but the role of the enzyme is still not clearly understood. The suggestion has been made (McLean, 1950) that phosphatase may be required for the synthesis of a specifically calcifiable matrix. Confirmation of its importance has been provided by the recently discovered disease, termed hypophosphatasia, which is characterized by remarkable changes in the bones, closely resembling those of rickets, and it is interesting that in this disease phosphoethanolamine has been found in the urine (McCance, Morrison & Dent, 1955; Fraser, Yendt & Christie, 1955). This is evidently one of the substrates upon which phosphatase may act in the body.

In histochemical studies of epiphyseal cartilage in rats, Morse & Greep (1951) found no evidence that the phosphatase activity of any particular histological element was altered in low phosphorus rickets. The extent of the phosphatase-positive areas was increased, but this was due to structural changes in the bone and particularly to the enlargement of the zone of hypertrophy, the cells of which are rich in phosphatase. No analogous studies appear to have been made on the bones of rachitic children, but it is well known that in human rickets there is a notable increase in serum phosphatase activity. A pronounced increase has not been observed in low phosphorus rickets of rats, but in many respects rickets in rats differs from the syndrome in children or dogs to which the name was first applied.

Harris (1932) and Glock (1940) showed that glycogen accumulates in cartilage cells and disappears in the early stages of calcium deposition, and they suggested that phosphoric acid esters formed in glycogen breakdown may act as a substrate for phosphatase. The presence of a phosphorylase in calcifying cartilage, capable of converting glycogen into phosphoric acid esters, was shortly afterwards demonstrated by Gutman & Gutman (1941), and some evidence was obtained, in experiments with enzyme inhibitors, that phosphorylase is necessary in calcification (Gutman, Warrick & Gutman, 1942). More recently, adenosine triphosphate (Albaum, Hirshfeld & Sobel, 1952a) and most of the glycogenolytic enzymes and several phosphorylated intermediates (Albaum, Hirshfeld & Sobel, 1952b) have been demonstrated in cartilage and, in addition, a protein-bound phosphate ester of a hexosamine, which may be concerned in calcification (Distefano, Neuman & Rouser, 1953). The presence in bone, as in other tissues, of enzyme systems capable of breaking down glycogen is thus well established, but whether glycogenolysis is necessary for the production of phosphorus in a special state of combination, as well as for energy liberation, is not yet clear. In a histochemical study of rachitic cartilage, Follis (1949) found glycogen only in the most recently matured hypertrophic cells, in the area where calcium is deposited in the early stages of healing.

The observation that the oral administration of citric acid or citrate can have a healing effect on rickets (Hamilton & Dewar, 1937; Shohl, 1937) was followed by the British discovery of a relatively high concentration of citric acid in normal bone, and a lower one in rickets (Dickens, 1941). These findings suggested that citric acid may be closely concerned with calcification, and they stimulated interest in citric acid metabolism. In a study of enzymes of the citric acid cycle in normal tissues, made at the Institute of Orthopaedics, London, Dixon & Perkins (1952) found that the citrogenase and aconitase activities of bone were relatively greater than its *iso*citric dehydrogenase activity, as compared with liver and kidney. They proposed that this excess of citric-acid forming enzyme (citrogenase), over citric-acid-removing enzyme (*iso*citric dehydrogenase), may represent the mechanism by which a high local concentration of citric acid is maintained in normal bone.

Harrison & Harrison (1952) showed that the administration of vitamin D to rachitic infants raised the concentration of citrate in the serum. Harrison later (1954) found elevated serum citrate concentrations in a variety of conditions in which diffuse demineralization of the skeleton occurs; this suggests that the blood citrate level may be related to bone resorption processes. In manometric studies of epiphyseal cartilage from rats given a rachitogenic diet with or without vitamin D, Tulpule & Patwardhan (1954) found no evidence that a deficiency of vitamin D led to any disturbance of the anaerobic phase of glycogenolysis, but that it reduced the oxidation of pyruvic acid. This opens up the possibility that the reduction of citric acid which has been reported in rachitic bone may be due to some fault in pyruvate oxidation.

d. Mode of Action

Although the mechanism of the calcification process in normal bone is still not fully understood, it is known that the serum concentrations of calcium and phosphorus, and particularly the product of these concentrations (the so-called Ca × P product), have an influence on the process. There is evidence that vitamin D can influence these levels by increasing the intestinal absorption of calcium and phosphorus and by increasing the tubular reabsorption of phosphorus by the kidney. It also appears that a "local factor" in bone is necessary for calcification as well as the Ca × P product of the serum and there are indications that vitamin D may have a direct effect upon the local mechanism.

Evidence that vitamin D promotes the absorption of calcium through the intestinal mucous membrane, and promotes phosphorus absorption secondarily through its effect on calcium absorption, has been summarized by Nicolaysen & Eeg-Larsen (1953) and Nicolaysen, Eeg-Larsen & Malm (1953). The mechanism of this effect is not known. Several dietary factors other than vitamin D can influence the onset and severity of rickets by interfering with calcium absorption. Thus, in addition to phytic acid, fatty acids and oxalic acid can reduce calcium absorption by the formation of insoluble compounds with calcium in the intestinal tract. The rachitogenic effect of green oats and other feeds upon lambs has recently been traced to the large amount of carotene in them (Grant, 1953). How carotene acts in precipitating rickets is not yet known, but the effect is antagonized by vitamin D.

The influence of vitamin D on the tubular reabsorption of phosphorus by the kidney was first observed by Harrison & Harrison (1941). It has not been proved whether this effect is a direct one on the tubules or whether it is secondary to a rise in the level of calcium in the serum and a consequential change in parathyroid activity. Harrison (1954) favours the view that vitamin D affects the tubule cells directly, and has shown that maleic acid and acetazoleamide can produce effects on the tubules resembling those seen in the Fanconi syndrome and hyperchloraemic renal tubular acidosis.

The identity of the local factor in bone which appears to be necessary for the initiation of calcification has not yet been

established, but it has been suggested that acid mucopolysaccharides such as chondroitin sulphate may play a part, through the accumulation of calcium by the acid groups. In experiments with ^{35}S, Dziewiatkowski (1954) obtained evidence that vitamin D may have a direct influence on calcification by interfering with the utilization of chondroitin sulphate in the skeleton.

Barnicot (1948) showed clearly that a high concentration of vitamin D can have a direct local effect on bone, as pieces of bone to which calciferol crystals had been attached, and which had been implanted in the cerebral hemispheres of mice, showed resorption at the site of the crystals. This finding prompted Barnicot to suggest that the osteoporosis of hypervitaminosis D may be due, at least in part, to a local action of the vitamin on bone. Some caution must be exercised, however, in correlating the effects of massive doses of the vitamin with those of physiological amounts, for the mechanisms of action at high and low levels may not be identical. Thus, Harrison (1954) produced evidence that dihydrotachysterol, which differs from calciferol in having a methyl group on the ring instead of a methylene group, and which has no appreciable antirachitic effect at low levels of dosage, was potent at high levels when administered to a patient with refractory rickets.

* * *

Although the exact parts played by vitamins A, C and D in skeletal development have as yet eluded investigators, it can be seen that knowledge has now ceased to be largely morphological and interest has begun to centre on problems of cell metabolism. The implication of acid mucopolysaccharides and phosphatase in both the scorbutic and rachitic syndromes indicates that they play important parts in bone growth and points to the need for fuller information about the normal biochemical processes involved in the formation of the matrix as well as in the deposition of the bone crystals.

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VITAMIN DEFICIENCY AND VISION

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1 Effect of vitamin A deficiency
2 Vitamin B deficiencies
  a Thiamine deficiency
  b Tropical nutritional amblyopia
  c Malnutritional keratoconjunctivitis
  d Riboflavin deficiency
3 Conclusions
References

Vision is the end result of a series of complex processes in eye and brain[1]; vitamin deficiency may cause obscurity of vision through impairment of light reception by the retina or impairment of the transmission of the stimulus to the brain. Animal experiments are handicapped by the fact that it is difficult to assess vision in the animal and it is true to say that our knowledge of the effect of vitamin deficiency on vision has come as much from observation in man as from experiments with animals.

1. Effect of Vitamin A Deficiency

It is well established that deficiency of vitamin A can cause night-blindness and, in cases of greater severity, the conjunctival and corneal changes of xerophthalmia and keratomalacia leading to perforation of the eye and blindness. A thorough examination of the conditions necessary to establish vitamin A deficiency in the human adult was made in Britain during the Second World War (Hume & Krebs, 1949). Contrary to expectation only three of the 16 deprived subjects showed any change in their dark-adaptation curve after 10, 12 and 20 months of deficiency. The sensitivity of the fully dark-adapted eye of one man fell to one-tenth and of another to one-hundredth of the value at the start (see Hume & Krebs, 1949, fig. 4), yet the two volunteers concerned thought that their night vision was only slightly impaired.

All the deprived subjects showed constriction of the visual field of the fully dark-adapted eye measured 10 months after the start by the method of Livingston (1944). Constriction of the field was shown by deprived subjects who had normal dark-adaptation curves. (See Hume & Krebs, 1949, figs. 8 & 9.)

Curative tests showed that 2,500 i.u. of vitamin A would restore plasma vitamin A and bring the dark-adaptation curve to normal. Conclusions that may be drawn are: (i) that it is exceedingly difficult to induce deficiency in a well-fed European adult; (ii) that the sensitivity of the eye to light can diminish to about 1% of the normal without the subject being at all seriously night-blind; and (iii) that cure may be achieved by relatively small doses of vitamin A. These findings indicate clearly that a profound deficiency must be necessary to produce xerophthalmia and keratomalacia. Yet until quite recently keratomalacia was considered by Wright (1942) to be the largest single cause of blindness in southern India.

The subjects in the Sheffield experiment showed a fall in blood vitamin A from about 120 i.u./100 ml. plasma to about 50 i.u./100 ml. plasma before any change in dark-adaptation was found (Hume & Krebs, 1949). Babies with severe corneal lesions due to vitamin A deficiency may have only 0–8 i.u./100 ml. plasma (de Haas & Meulemans, 1938). An experiment in which change in plasma vitamin A was correlated with conjunctival and corneal lesions in the rabbit showed that the vitamin A of the plasma fell from about 160 i.u./100 ml. to about 10 i.u./100 ml. before epithelial change in the cornea could be detected clinically (Mann, Pirie, Tansley & Wood, 1946).

Charpentier (1936) found a reduction in amplitude of the electroretinogram in the vitamin-A-deficient rat and this has been confirmed in the rabbit (Waters, 1950) and in a man (Bornschein & Vukovich, 1953). A rise in the final rod threshold, measured by the electroretinogram, could be detected in vitamin-A-deficient rabbits before any corneal signs were visible. Vitamin A restored the electroretinogram to normal. This objective method of measuring retinal function may be of value in the study of other nutritional deficiencies.

Recent work on structural change in the eye due to vitamin A deficiency has centred on changes in the young animal. Warkany (1954) has found that severe maternal vitamin A deficiency in the rat induces retinal and other ocular malformation in the young. Calves (L. A. Moore, 1939) and rabbits (Mellanby, 1944) deficient in vitamin A may go blind owing to distortion of growth of the bone surrounding the optic foramen, with consequent constriction of the optic nerve.

2. Vitamin B Deficiencies

a. Thiamine Deficiency

There is fairly clear evidence that thiamine deficiency is the chief cause of tobacco-alcohol amblyopia and of the ocular signs of Wernicke's encephalopathy.

Tobacco-alcohol amblyopia. This is characterized by bilateral impairment of central vision, with development of centro-caecal scotomata. The end result is degeneration of the ganglion cells of the retina and fibres of the optic nerve, particularly those of the papillo-macular bundle. Cases of simple tobacco-amblyopia are commonest in Britain; cigarette smokers are unaffected—it is the smokers of pipes and cigars who develop amblyopia. If structural damage has not occurred, there is improvement if tobacco and alcohol are renounced. But Carroll (1944) found that improvement may also be achieved by giving thiamine although the subject continues to drink and smoke. There is, however, some evidence that vision is restored more rapidly and completely if all B vitamins are given. This is understandable since deficiencies are seldom simple in man.

Wernicke's encephalopathy. This disease was originally thought to be the result of chronic alcoholism but has more recently been described in prisoners of war and those not addicted to alcohol. The eye signs are nystagmus and paralysis of the ocular muscles. De Wardener & Lennox (1947) described 52 cases in a camp for British prisoners of war in Singapore. Every case showed nystagmus and some showed

[1] A recent number of the Bulletin (*Brit. med. Bull.* 1953, Vol. 9, No. 1) was devoted to the "Physiology of Vision".—ED.

ophthalmoplegias. Spillane (1947) reported cases among prisoners of war in the Middle East. Thiamine was found to cure both nystagmus and ocular muscle paralyses. Later, Phillips, Victor, Adams & Davidson (1952), in a controlled series of civilian cases, found that thiamine alone was curative, other members of the B complex without thiamine being inactive. The ocular muscle paralyses were cured in a few hours, and the nystagmus disappeared within a few days. Such rapid reversal is almost certainly a reversal of a biochemical lesion rather than cure of a structural one. Cases of Wernicke's disease show increased blood pyruvate, indicative of a lack of cocarboxylase in the tissues (Wortis, Bueding, Stein & Jolliffe, 1942). Thiamine administration leads to formation of the coenzyme cocarboxylase and thus to restoration of normal metabolism.

b. Tropical Nutritional Amblyopia

Medical officers from many parts of the world have reported a failure of vision which they have ascribed to nutritional causes. The symptoms are a gradually developing inability to see clearly in daylight, and inability to read. Night vision is unaffected. Failure of vision may be the only sign or there may also be signs of deficiency of riboflavin, thiamine or pantothenic acid. Such nutritional amblyopia has occurred in prisoners in the Malay States (Landor & Pallister, 1935), in schoolchildren in Nigeria (D. G. F. Moore, 1939), in the West Indies (Metivier, 1941), in India (Raman & Abbu, 1940) and in prisoners in the Middle East (Spillane, 1947). The largest number of cases occurred in prisoner-of-war camps under Japanese control (Smith & Woodruff, 1951; Livingston, 1946; Ridley, 1946). In one camp there were 1,153 cases during 18 months.

The retina appeared normal ophthalmoscopically in most cases, but examination of the visual fields showed bilateral central scotomata of 4–5° (visual acuity fell in many cases to 6/60). Dark-adaptation was normal. The pre-war reports suggested that improvement was obtained by treatment with preparations of the vitamin B complex, if this was started in the early stages. In the prisoner-of-war camps heroic efforts were made to find what the nutritional cause might be. There seemed no connexion between incidence of visual failure and thiamine intake. On the other hand, there did seem to be a relation to deficiencies of the vitamin B complex. Treatment of 10 early cases with a diet containing considerable riboflavin (as leaf extract) caused marked improvement in the fourth week of treatment.

There is still no certainty of the cause of this amblyopia. Rodger (1952) analysed the cases of prisoners returned from the Far East and found a statistically significant association with beriberi. After release, 38% got worse, 33% were unchanged and 29% improved. In general the evidence supports the view that tropical nutritional amblyopia is due to deficiency of one or more vitamins of the B complex, but a toxic factor has also been suggested.

These three disorders of vision—tobacco-alcohol amblyopia, Wernicke's disease and tropical nutritional amblyopia—are known only in man. Animal experiments designed to show whether deprivation of thiamine or riboflavin affects the retina or optic nerve have been few and conflicting. But it does appear that, if a chronic thiamine deficiency can be established, pathological changes can be detected histologically in the central terminations of the optic nerve fibres of the pigeon (Swank & Prados, 1942) and of the rat (Rodger, 1953). Brain lesions, which are distributed exactly as in cases of Wernicke's disease, occur in the chronically thiamine-deficient pigeon (Alexander, 1940).

Rodger (1954) showed that, although no change could be detected in the visual pathway of rats made chronically deficient in riboflavin, a combined deficiency of riboflavin and thiamine caused grosser pathological change than the deficiency of thiamine alone. It is possible that such a combined deficiency is behind the development of tropical nutritional amblyopia.

c. Malnutritional Keratoconjunctivitis

This is a blinding disease which has been observed by Blumenthal (1950, 1954) in the young Bantu in the eastern Cape Province of South Africa. (See Pl. I, fig. A.) It is considered to be the largest single cause of blindness in Bantu children, over 150 cases having been seen in two years in the out-patient clinic of one hospital. The disease can take many forms, which makes it probable that it has a complex origin. The most characteristic presenting sign is a small perforation in the cornea, through which the iris prolapses. The eye is not inflamed. This form is seen in infants and young children only; older children show various changes in the central cornea, leading to softening, change of shape, and rupture. In some cases secondary infection occurs and becomes the dominating feature. If the cornea does not rupture, scar tissue forms, and the cornea becomes thickened and opaque. The child is abnormal, being listless, with rough hair, dry skin and distended belly. The children who contract the disease are those who live mainly on mealie-meal after weaning.

> Not until the toddler goes foraging for himself with older children for roots and berries, birds and their eggs, small animals and insects, does he find the necessary vitamins, or . . . trace elements. In locations and towns this foraging is often on rubbish dumps, etc., where meat, fruit and vegetable refuse may be found [Blumenthal, 1954].

No clear idea of the cause has yet been given, but it is certain that the condition of the eyes improves with good food or with multivitamin treatment. Since the first stage appears to be softening of the corneal stroma, whereas the corneal epithelium is normal; and since the eye is wet, not xerotic, malnutritional keratoconjunctivitis is considered to be a separate disease from the keratomalacia of vitamin A deficiency.

d. Riboflavin Deficiency

Corneal vascularization due to riboflavin deficiency was first described in the rat (Day, Langston & O'Brien, 1931; Bessey & Wolbach, 1939). It is a bilateral symmetrical invasion of the cornea by capillaries which lie just beneath the epithelium. The cornea is also invaded by cells which appear to precede the capillaries. Corneal vascularization of much the same type as that seen in the rat was first reported in riboflavin-deficient men in 1940 by Sydenstricker, Sebrell, Cleckley & Krause.

Riboflavin coenzymes occur in all the tissues of the eye in rather low concentration (Philpot & Pirie, 1943). Bessey & Lowry (1944) found that the corneas of riboflavin-deficient rats soon lose most of their riboflavin and that after three weeks' deprivation the riboflavin of the cornea had dropped to about 40% of the normal. At this time vascularization sets in and thereafter the riboflavin of the cornea remains at this

level. The biochemical change which leads to ingrowth of capillaries is unknown.

3. Conclusions

There is little ocular disease caused by uncomplicated vitamin deficiency in Europe today; but if we take the world as a whole the picture is different, as the report on colonial nutrition (Committee on Nutrition in the Colonial Empire, 1939a, 1939b) clearly sets out. Reports from many parts of the world show that malnutrition, particularly vitamin A deficiency, leads to much preventable eye disease. Our knowledge is very incomplete. The brief survey given in this article describes two diseases of the eye, tropical nutritional amblyopia and malnutritional keratoconjunctivitis, whose exact cause we do not know, although we can say with fair certainty that they are nutritional in origin.[2] It is probable that, as further surveys are made, conditions of the eye not now considered to be related to malnutrition will be found to have this as a contributory factor or main cause.

[2] For some discussion of retrolental fibroplasia and vitamin E deficiency, see p. 47 of the paper by Moore in this number of the Bulletin.—ED.

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VITAMIN DEFICIENCY AND VISION

(FIG. A)

Antoinette Pirie

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Blumenthal (1950) by permission of South African Medical Journal

FIG. A. Malnutritional keratoconjunctivitis, showing very recent spontaneous iris prolapse. Some organization is already taking place in margin of cornea surrounding prolapse

VITAMIN A AND TISSUE CULTURES

(FIGS. B – D)

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a

b

FIG. B. Effect of excess vitamin A on a radius from a mouse foetus at a late stage of gestation

a: radius after cultivation for 9 days in normal medium. Note the bony diaphysis with marrow cavity and cartilaginous ends (magnification × 25)

b: opposite radius from same foetus, grown for 9 days in medium containing about 1,000 i.u./ml. of added vitamin A. The terminal cartilage has nearly disappeared and there is extensive resorption of diaphyseal bone (Stain: Delafield's haematoxylin and chromotrop 2R; magnification × 25)

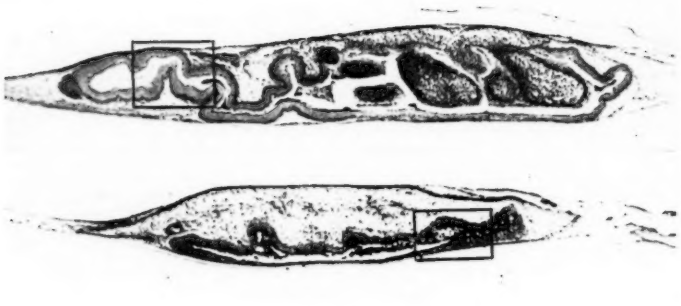

FIG. C. Effect of excess vitamin A on skin from 7-day-old chick embryo

a: skin after cultivation for 10 days in normal medium. The ectoderm, which has formed a cyst, has differentiated into squamous epithelium, which is keratinizing profusely (magnification × 50)

b: skin from opposite side of same embryo after cultivation for 10 days in medium containing about 2,000 i.u./100 ml. of added vitamin A. The epithelium has differentiated into an actively secreting mucous membrane; the lumen of the cyst is filled with secretion positive by the periodic acid-Schiff method (Stain: periodic acid-Schiff method and Meyer's acid haemalum; magnification × 50)

FIG. D. Ruled sections of explants in fig. C under higher magnification

a: control grown in normal medium, showing squamous, keratinizing epithelium

k: keratin (magnification × 350)

b: explant grown in high A medium (about 2,000 i.u./ml. of added vitamin A). The epithelium consists of a basal layer of small cubical cells and a columnar secretory stratum

m: mitosis
p: pigment (melanin)
s: secretion
s.c.: actively secreting cell
(magnification × 350)

EFFECT OF EXCESS VITAMIN A ON ORGANIZED TISSUES CULTIVATED IN VITRO

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1 Explanted bone rudiments in vitamin-enriched media
2 Vitamin A effects on ectoderm
3 Interaction of vitamin A and a carcinogen
4 Sulphur metabolism in tissues examined by autoradiography in media labelled with ^{35}S
References

Elsewhere in this number of the Bulletin, an account has been given of Sir Edward Mellanby's work on the effect of vitamin A deficiency on the bones of young animals.[1] It occurred to Mellanby that further information about the action of the vitamin might be obtained by tissue-culture experiments. This idea proved to be very fruitful, and a series of studies were made on the effects of hypervitaminosis A on organized tissues growing in vitro. The results of this work are reviewed in the present article.

Other investigators have shown (for a brief survey of the literature see Fell & Mellanby, 1953) that, when young animals are fed on a diet containing excess vitamin A, the skeleton is severely affected; there is rapid resorption of the cartilage, and rarefaction of the bone often leads to spontaneous fractures. It was uncertain whether these effects were due to a direct action of the vitamin on the skeleton or were mediated through some other system or organ of the body. The first experiments undertaken by Mellanby and myself (Fell & Mellanby, 1952) were designed to show whether the vitamin, in concentrations similar to those that could be produced in the blood of hypervitaminotic animals, would affect entire bone rudiments grown in culture where all indirect actions due to other parts of the body would be eliminated.

1. Explanted Bone Rudiments in Vitamin-Enriched Media

Isolated long-bone rudiments from mouse foetuses at a late stage were grown by the watch-glass method described by Fell & Robison (1929). One of each pair of rudiments was explanted in normal medium consisting of plasma and embryo extract, and the other in the same medium to which had been added 1,500–2,000 i.u. of vitamin A/100 ml. (high A medium). The controls enlarged slightly and remained healthy and intact throughout the maximum culture period (10 days), but the bones in high A medium were drastically changed (Pl. I, fig. B). The terminal cartilage lost its metachromasia, shrank and finally disintegrated, whereas the bone of the shaft became increasingly rarefied; with a dose of about 2,000 i.u./10 ml., the explant had nearly disappeared by the 10th day. These severe changes were not accompanied by extensive cell degeneration in the bone and cartilage, and, for the dissolution of the intercellular material, living cells were necessary; if the rudiments were killed by heating to 45° C. before explantation, the vitamin had no effect. Excess vitamin A had a similar action on explants of the cartilaginous long-bone rudiments of six-day-old chick embryos; in these cultures also the cartilaginous matrix lost its metachromasia, shrank and eventually almost or completely disappeared. Recently Giroud & Martinet (1954) have described, amongst other abnormalities, considerable retardation of the growth and development of the cartilage bones in the foetuses of pregnant rats suffering from hypervitaminosis A.

Although the skeletal tissue was so adversely affected by the vitamin, the surrounding soft tissue grew more profusely in the high A than in the normal medium. This observation agrees with the results of Baker (1935), who found that moderate doses of vitamin A greatly increased the outgrowth of chick fibroblasts, although high concentrations were toxic. Recently Lasnitzki (1955a, 1955b) has shown that doses of vitamin A which caused disruption of cartilage and bone in vitro stimulated mitosis in fibroblastic cultures.

When chick-bone rudiments which had been grown for several days in high A medium were transferred to a normal clot (Herbertson, 1955), the changes produced by the hypervitaminosis progressed at a diminishing rate for some days and were then arrested. During the period of the experiments, the altered cartilage did not recover its normal appearance; metachromasia which had been lost was not regained, and diminished matrix was not restored. The small-celled epiphyses enlarged after transfer to normal medium, but this appeared to be owing to very active mitosis among the chondroblasts and not to the formation of new metachromatic matrix. After the transfer, periosteal ossification was often very profuse.

The results described above were all obtained with an " artificial " hypervitaminosis produced by adding the vitamin to normal plasma. The effects were compared with those of " natural " hypervitaminosis in which the plasma was drawn from a fowl fed with excess vitamin A. It was found that vitamin A, in the same concentration, was much more potent in the artificial than in the natural high A medium, although the type of effect produced was the same in both. This difference in activity was correlated with a difference in the extractability of the vitamin which could be removed almost completely from the artificial high A plasma with light petroleum, whereas it could be recovered in this way from the natural high A plasma only after the proteins had been denatured with 50% (v/v) ethanol.

2. Vitamin A Effects on Ectoderm

One of the most familiar effects of vitamin A deficiency in vivo is the squamous metaplasia and keratinization of certain mucous membranes. Experiments were made (Fell & Mellanby, 1953) to see whether the reverse effect could be produced in vitro by hypervitaminosis A. When the ectoderm from the trunk or limbs of chick embryos six to seven days old is grown in normal medium for about a week, it develops into a squamous, keratinizing epithelium (Pl. II, figs. Ca, Da); the object of Fell and Mellanby's experiments was to see whether this keratinization could be inhibited by adding excess

[1] See Holman & McCance, p. 27 of this number of the Bulletin.—Ed.

vitamin A to the medium. It was found that the addition of 1,500–2,000 i.u./100 ml. of the vitamin not only inhibited keratinization but transformed the presumptive epidermis into an actively secreting mucous membrane which was often ciliated; the epithelium (figs. Cb, Db) somewhat resembled that of the normal nasal mucosa which also is of ectodermal origin. As in the controls, feather papillae differentiated and pigment cells wandered into the ectoderm (fig. Db); feather germs and beating cilia were sometimes seen in the same cyst. When the explants were transferred from high A to normal medium, the basal cells multiplied and formed a squamous, keratinizing epithelium beneath the secretory layer which degenerated and was shed.

Vitamin A is taken up very quickly by the ectodermal cells; this was demonstrated by the interesting experiments of Weiss & James (1954, 1955). Moscona (1952) has shown that if an embryonic tissue is broken down by enzymic digestion into a suspension of single cells which are then allowed to re-aggregate and are grown as an organ culture, they redifferentiate into a tissue resembling that from which they were derived (Moscona & Moscona, 1952). Using a similar method, Weiss and James reduced the skin of the embryonic chick to a cell suspension, part of which was then immersed in a 0.06% solution of vitamin A in 6% ethanol in Tyrode's solution for 30–60 min.; the cells were thoroughly washed, allowed to re-aggregate and cultivated in normal medium. Control cells were treated in exactly the same way, except that they were immersed in 6% ethanol only, before re-aggregation. In the controls, the re-aggregated cells formed squamous, keratinizing epithelium in which feather germs developed, but the cells treated with vitamin A produced epithelium of a secretory type in which keratinization was inhibited. If the tissue was again treated with a solution of vitamin A at subculture, mucus-secreting cells developed similar to those which appeared in Fell and Mellanby's cultures grown in high A medium.

Two other ectodermal derivatives, viz. the retina and otocyst of four-day-old embryonic chicks, underwent no metaplastic changes in high A medium; in the presence of excess vitamin A the retina grew better, while the formation of a cartilaginous capsule around the otocyst was completely suppressed (Fell & Mellanby, unpublished data), but histologically both rudiments differentiated normally.

Kahn (1954) found that organ cultures of the vagina responded to vitamin A. If explants of the vagina of young rats were maintained in chicken plasma and embryo extract for about six days, the epithelium keratinized, but if the medium was supplemented with vitamin A, keratinization was inhibited and the cells retained their secretory character. A dose of vitamin A, sufficient to prevent keratinization in normal medium, did not stop its appearance when a suitable concentration of oestrogen was added to the medium.

3. Interaction of Vitamin A and a Carcinogen

Lasnitzki (1955a) has studied the interaction of a carcinogen (20-methylcholanthrene) and vitamin A on cultures of the prostate gland of young mice. When grown in chicken plasma and embryo extract, such explants remained healthy and retained their normal histological structure for several weeks. When cultivated for 10 days in the same medium to which methylcholanthrene had been added, the epithelium became hyperplastic, lost its columnar, secretory structure and finally underwent squamous metaplasia and keratinization, a condition which was maintained for 10 days or more after the explants had been transferred to normal medium. If vitamin A (1,500 i.u./100 ml. of medium) was added to the cultures at the same time as the carcinogen, the epithelium became hyperplastic, but squamous metaplasia and keratinization were inhibited, and the cells next the lumina of the alveoli retained their secretory character. If the vitamin was introduced after treatment with the carcinogen, there was not only a return to the secretory condition but the hyperplasia was reduced.

4. Sulphur Metabolism in Tissues Examined by Autoradiography in Media Labelled with ^{35}S

It occurred to S. R. Pelc that the changes produced by vitamin A in cartilage and chick ectoderm might be correlated with an altered sulphur metabolism. To investigate this possibility, the uptake of radioactive sulphur, applied in the form of sodium sulphate, by cultures grown in high A and in normal medium was studied by autoradiography, the stripping-film method being used (Doniach & Pelc, 1950).[2] Chick long-bone rudiments grown in normal medium rapidly took up $^{35}SO_4$ (Pelc, Mellanby & Fell, unpublished data) which was at first concentrated largely in the cells of the still actively growing part and then became evenly diffused throughout the matrix which retained all or most of the incorporated sulphate during subsequent cultivation on unlabelled medium. During the first four days in the presence of excess vitamin A, the cartilage was able to take up $^{35}SO_4$, but when the explants were transferred to unlabelled high A medium, much of the sulphate was lost after two days. After six days in excess vitamin A, the peripheral cartilage and the entire middle segment of the diaphysis ceased to incorporate the isotope. There was a close correlation between failure to take up sulphate and loss of metachromasia in the cartilaginous matrix.

It was also shown (Fell, Mellanby & Pelc, 1954) that, when the presumptive epidermis of the chick was transformed into mucus-secreting epithelium by hypervitaminosis A, its sulphur metabolism was profoundly modified. A small but significant amount of sulphate was taken up by the squamous, keratinizing epithelium of the controls, which thus behaved like normal epidermis in vivo (Glucksmann, Howard & Pelc, unpublished data), whereas in explants grown in high A medium, the autoradiograph of the inner secretory cells was dense, indicating that the utilization of sulphate was comparable to that of a normal mucous membrane in the body.

These investigations on the effect of vitamin A on organ cultures in vitro have shown that the vitamin has a direct action on a wide variety of tissues grown under conditions which preclude indirect effects mediated through other systems or organs. The autoradiographic experiments have demonstrated that the vitamin radically affects the sulphur metabolism of the tissues in a manner which is not yet properly understood. In the ectoderm the vitamin causes the cells to synthesize mucus; it may be tentatively suggested that perhaps the cartilage cells are similarly affected and that, instead of synthesizing chondroitin sulphate capable of forming a stable matrix, they may secrete a more soluble form similar to that contained in mucus. Further work is required to see whether there is any truth in this idea.

[2] See also Pelc & Howard, *Brit. med. Bull.* 1952, 8, 132.—Ed.

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CAROTENOIDS AND VITAMIN A

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1 Carotenoid precursors and vitamins A
2 Analytical chemistry and standards
3 Conversion of carotene to vitamin A
4 Vitamin A and vision
5 Systemic action
References

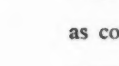
Sir Edward Mellanby in his Croonian Lecture in 1943 described vitamin A and vitamin D as the David and Jonathan of nutrition, and his eloquent parable of the beleaguered city illustrated the balancing of osteoblastic and osteoclastic processes (Mellanby, 1938, 1941, 1944).

Throughout his period at the Medical Research Council, he maintained his interest in the work of the Vitamin A Subcommittee[1], and after his retirement he continued, with Dr Honor Fell[2], to study the mode of action of vitamin A, particularly in hypervitaminosis. Mellanby gave wise and sustained support to efforts to unify biological standardization and physico-chemical determinations of vitamin A in liver oils. He showed the greatest interest in establishing human requirements and thought it part of his job " to get the vitamin A down the throats of the people who need it ".

The subject of vitamin A and its mode of action is a very large one with an extensive literature. A short review cannot include all the important references or achieve a just emphasis; the bias here is on work which has influenced thought in Britain.

1. Carotenoid Precursors and Vitamins A

Carotenoids are pigments of plant origin; there is no evidence that they can be synthesized from simple compounds by animals. Some lower animals are, however, able to effect slight alterations (mainly by oxidization) in the structures of ingested carotenoids before accumulation occurs in liver, skin or gonads.

The structural formulae of the principal carotenoids are now firmly established mainly as a result of the work of Karrer and Kuhn and their collaborators. The general formula for carotenoids is $C_{40}H_{56}O_n$ where n is 0–6; hydrocarbons ($n = 0$) are termed " carotenes ", and oxygenated carotenoids " xanthophylls ". Carotenoid molecules can be looked upon as consisting of eight $C_{(5)}$ (isoprene, ) units, arranged as if two $C_{(20)}$ units, produced by the head-to-tail condensation of four $C_{(5)}$ units, are finally joined tail to tail. This characteristic symmetry near the centre of a carotenoid molecule is the basis of an internationally agreed definition (cf. Karrer, 1948):

> Les caroténoïdes sont des pigments de couleur jaune jusqu'à rouge, de structure aliphatique ou aliphatique-alicyclique, lesquels se composent de groupes isoprène (le plus souvent 8), qui s'enchaînent de telle façon que les deux groupes méthyle à proximité du centre de la molécule sont en position 1, 6, tandis que toutes les autres chaînes latérales méthyle sont en position 1, 5. . . .

This definition makes it clear that visual pigments containing retinene$_1$ or retinene$_2$ should not be called carotenoid proteins (cf. Wald, 1954a; Willmer, 1953).

The structures shown below illustrate the principal $C_{40}H_{56}$ isomers and the numbering of carbon atoms (see fig. 1). β-Carotene, contains two β-ionone residues; α-carotene has one α-ionone and one β-ionone residue; γ-carotene has one β-ionone and one ψ-ionone residue, whereas lycopene is an open chain compound with two ψ-ionone residues. All four hydrocarbons have the same structure from $C_{(7)}$ to $C_{(7')}$.

The oxygen atoms of xanthophyll are generally present in either hydroxyl or keto groups at positions 3:3′ or 4:4′; two types of epoxide, however, also occur: (i) 5:6-epoxides and (ii) 5:8-epoxides in which a furan ring has been formed.

Carotenoids occur in all green tissues of plants (see Goodwin, 1952); β-carotene, the most important precursor of vitamin A, comprises from 10% to 30% of the total pigment; it is present inter alia in carrots, in red palm oil and in many edible berries and seeds, where it is often accompanied by cryptoxanthin (3-hydroxy-β-carotene), another vitamin A precursor. The carotenoids of animal tissues are not generally rich in provitamins A, although a little β-carotene is found in eggs and yellow body fat.

The vitamin A_1 molecule ($C_{20}H_{29}OH$) can be regarded structurally as derived from β-carotene by fission at the central double bond and addition of water; vitamin A_2 ($C_{20}H_{27}OH$), which occurs in many fish-liver oils, is 3-dehydrovitamin A_1, but it has no known direct carotenoid precursor. (See fig. 2.)

The aldehydes (XCHO) derived from vitamins A_1 and A_2 can be prepared by allowing a solution of the free vitamin $X.CH_2OH$ in light petroleum to stand over manganese dioxide (Morton & Goodwin, 1944; Ball, Goodwin &

[1] See Chick & Hume, p. 5 of this number of the Bulletin.—Ed.
[2] See Fell, p. 35 of this number of the Bulletin.—Ed.

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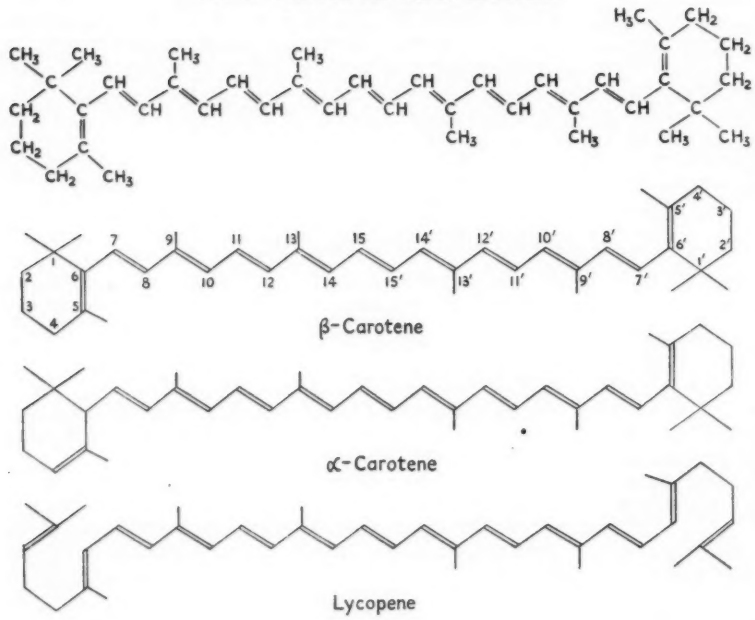
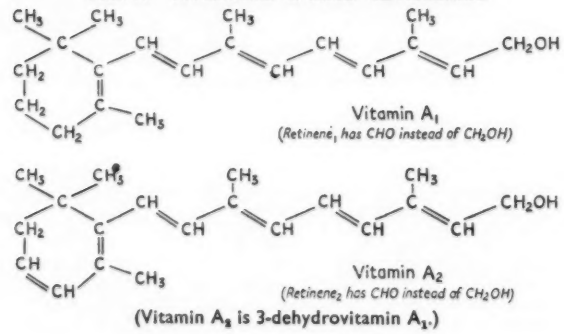
FIG 1. PRINCIPAL $C_{40}H_{56}$ ISOMERS



Morton, 1948). The spectroscopic properties of the two pure aldehydes retinene$_1$ and retinene$_2$ (Cama, Dalvi, Morton, Salah, Steinberg & Stubbs, 1952) agree with those recorded by Wald in his pioneer work (1934, 1935–36) on the breakdown of visual purples. As many as 1,000 cattle retinas dissected out in a dim red light are required in order to obtain about 1 mg. of retinene (Wald, 1954a); there is therefore a great advantage in having readily available retinenes.

Exploratory work on the isomerization of β-carotene by Gillam & El Ridi (1936) was ably followed up by Zechmeister (1944, 1954) and his colleagues, who worked out a whole range of interesting problems connected with *cis-trans* isomerism in the carotenoid field. Not only can the various provitamins A exist in isomeric forms, each with its own potential biological activity, but in addition there are also five retinene isomers. Hubbard & Wald (1952) have shown how one of these has a specific role in visual chemistry (see section 4 below).

FIG. 2. VITAMINS A AND RETINENES



(Vitamin A$_2$ is 3-dehydrovitamin A$_1$.)

In animal species vitamin A is stored, mainly as ester, in the liver, and to a lesser extent in the kidney. A small amount of free vitamin A alcohol is continuously liberated from the liver into the blood stream. This is perhaps the functional form, although the cure of avitaminosis A by vitamin A acid or methyl ether raises doubts as to whether this view is strictly correct.

2. Analytical Chemistry and Standards

Research in Britain has been much concerned with spectrophotometric analysis and with biological assays of liver oils. Both types of study have greatly facilitated investigations into their structure and synthesis.

The arsenic trichloride colour test (Rosenheim & Drummond, 1925) led to the antimony trichloride test (anhydrous $SbCl_3$ in chloroform (Carr & Price, 1926)) which permits the detection and spectroscopic differentiation of vitamin A_1 and A_2, the former giving a blue colour and the latter a greenish blue colour with the reagent (see Tables I, II). The recognition that vitamin A_1 had its own characteristic ultra-violet absorption ($\lambda_{max.}$ 328 mμ.) led to an alternative method of analysis, which, in first approximation, agreed well both with the colour test and the results of biological assays. The first standard substance was "carot-

TABLE I. ABSORPTION MAXIMA OF ALL-*TRANS* VITAMIN A

(Potency of all-*trans* vitamin A alcohol = 3.33 i.u./g. by definition)

Solvent	Vitamin A acetate			Vitamin A alcohol		
	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$	CF*	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$	CF*
*cyclo*Hexane	327.5	49,700	1,900	326.5	49,800	1,900
Ethanol	325–326	51,200	1,850	324–325	52,000	1,825
*iso*Propanol	326	50,300	1,900	325	52,400	1,825
$SbCl_3$ reagent	620	145,000				

* CF (conversion factor) $\times E^{1\%}_{1\ cm.}$ at $\lambda_{max.}$ = potency in i.u./g.

TABLE II. PROPERTIES OF NEOVITAMIN A AND VITAMIN A_2

Solvent	Neovitamin A		Vitamin A_2	
	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$
*cyclo*Hexane	328	47,200	351	*ca* 40,000
Ethanol	328	49,200	350	41,500
$SbCl_3$ reagent	620	*ca* 150,000	693	116,000

ene", chosen (League of Nations Health Organisation, 1931) largely because of the accidental circumstance that crystalline carotene was available earlier than crystalline vitamin A. This provisional standard was soon replaced (League of Nations Health Organisation, 1934) by a new preparation of pure β-carotene, the potency of which was fixed by definition as 1.66×10^6 i.u./g. Crystalline vitamin A (all-*trans* alcohol) turned out to be almost exactly twice as potent, and the unit of activity was redefined (World Health Organization, 1950) so that 0.3 μg. of vitamin A (or its stoicheiometric equivalent of acetate) showed one unit of activity. From the physical properties of pure vitamin A alcohol or acetate, conversion factors could be deduced to convert spectroscopic findings into international units. Conversion factors rather than a single conversion factor are necessary because the absorption spectra of equimolar solutions of vitamin A alcohol and acetate are not quite identical in a given solvent and there are also small variations from one solvent to another.

The problems which face analysts are still, however, quite complicated. Most of the fish-liver oils of commerce come from salt-water fishes and, although vitamin A_1 greatly preponderates over vitamin A_2, both are present. The ratio A_1: A_2 is not constant from species to species or even within a species. Also, the vitamin A_1 is a mixture of about three parts of the all-*trans* isomer and one part of neovitamin A, a *cis* isomer. In the main, vitamin A occurs as esters, but some oils contain a small proportion of free vitamin A, often accompanied by appreciable amounts of decomposition products, including epoxides, anhydrovitamin A and even traces of retinene. The intensity of absorption at 325–328 mμ. is the sum of that due to vitamin A and any other absorbing substances (conjugated unsaturated acids, vitamin A decomposition products, etc.) which may be present. In many fish-liver oils of high potency the contribution of all constituents other than vitamin A_1 (the irrelevant absorption) is a small fraction of the total absorption, but in oils of low potency less than half the gross absorption near 328 mμ. is due to vitamin A. The irrelevant absorption is reduced by preparing and examining unsaponifiable fractions (e.g. of cod-liver oil) and is to a great extent overcome by a simple geometrical correction procedure described by Morton & Stubbs (1946). This rests upon the assumption that the absorption exerted by impurities is linear over a comparatively small wavelength range from 310 mμ. to 340 mμ. For some materials, however, the only satisfactory procedure is to carry out a preliminary chromatographic purification prior to spectrophotometry. The correction method of Morton & Stubbs (1946) has been adopted in slightly different forms by the *Pharmacopeia of the United States of America* (1950) (U.S.P. XIV) and the *British Pharmacopoeia, 1953*. Because of the acceptance of too high a conversion factor for solutions of vitamin A alcohol in *iso*propanol, the U.S.P. XIV method gives results about 5% higher than the 1953 *British Pharmacopoeia* method for which the solvent is *cyclo*hexane.

3. Conversion of Carotene to Vitamin A

The first proof that carotene is a vitamin precursor was obtained by Moore (1931) who recorded an increase in the vitamin A liver stores of rats receiving carotene. It was natural to assume that the liver was the site of the conversion, but a considerable number of very careful experiments failed to confirm this.

An interesting discovery (Lovern, Edisbury & Morton, 1937) showed that many species of fish possess large stores of vitamin A in the lipid of the mucosae of the pyloric caecae and small intestines. It is not known whether the enormous accumulation of vitamin A in the absorptive part of the gut of halibut and other species is the result of a failure to transport the ingested vitamin A or whether the mucosae are the site of a provitamin to vitamin conversion (Edisbury, Morton, Simpkins & Lovern, 1938). Although vitamin-rich intestinal oils were soon being prepared commercially, the theoretical implications are even yet not clear.

Soon after the Second World War three groups of workers reached the conclusion that the site of conversion of β-carotene into vitamin A in the rat was the intestine. Sexton, Mehl & Deuel (1946) argued that " the conversion of carotene to vitamin A may be an extra-hepatic function in the rat. The wall of the intestine is suggested as a possible site of such transformation". Glover, Goodwin & Morton (1947, 1948b), Mattson, Mehl & Deuel (1947), and Thompson, Ganguly & Kon (1947, 1949) demonstrated that such was the case.

Ball, Glover, Goodwin & Morton (1947) and Glover, Goodwin & Morton (1948a) had shown that retinene$_1$ (vitamin A_1 aldehyde), administered to rats orally, subcutaneously or intraperitoneally, is converted to vitamin A_1 during absorption. After oral administration the conversion takes place in the gut wall, but after subcutaneous injection it occurs near the site of injection. This implies the action of a wide-spread alcohol dehydrogenase and raises the question whether retinene is an intermediate between carotene and vitamin A.

Various later experiments indicate a predominant role for the intestine as the site of vitamin A formation. Thus ligation of most of the blood vessels leading to the liver did not impair the conversion of orally ingested carotene to vitamin A (Krause & Pierce, 1948), moreover incubation of carotene (dispersed in water) in an isolated loop of rat intestine was found to result in formation of vitamin A at a rate fast enough to account for the conversion which occurred after administering the dispersion by stomach tube (Rosenberg & Sobel, 1953). The intestine is not, however, the exclusive site of the process. By injecting a carotene suspension into avitaminotic calves, Eaton, Matterson, Decker, Helmboldt & Jungherr (1951) obtained a small increase in plasma vitamin A without any change in the liver level. Bieri & Schultze (1951) gave to rats depleted of vitamin A a carotene dispersion by intramuscular injection; they found vitamin A in plasma and kidney but not in liver. Bieri & Pollard (1954) showed that carotene given intravenously in the detergent Tween 40 restored growth to depleted rats; removal of the small intestine did not impair the formation of vitamin A from injected carotene; and nephrectomized and partially hepatectomized rats could also utilize injected carotene.

There can, however, be little doubt that carotene (especially in the presence of tocopherol) is in most species moderately well absorbed and is converted into vitamin A in the intestine. There is also evidence of differences between species and of the diminished absorption of excess of carotene.

The primary process in the formation of vitamin A thus seems to be an oxidative fission of a carotenoid precursor which, if it were restricted to the central (15:15′) double bond, could yield two molecules of retinene per molecule of β-carotene; each of these could be reduced to vitamin A. The " theoretical " conversion has been claimed by some

workers, but all the experience gained in the course of collaborative bio-assays (including those organized by the Vitamin A Subcommittee of the Medical Research Council's Accessory Food Factors Committee) pointed to the equivalence of 0.3 μg. of vitamin A and 0.6 μg. of β-carotene. This would follow from a mechanism of β-oxidation following oxidative fission at the double bond in one of the two β-ionone rings. Oxidation would not then be specific to the 15:15′ double bond, but retinene$_1$ would still be the immediate precursor of vitamin A. Glover & Redfearn (1954) have provided interesting evidence in favour of this view.

The absorption of β-carotene varies directly with thyroid activity in rat (Cama & Goodwin, 1949), goat and cow (Chanda, Clapham, McNaught & Owen, 1951). Alternative explanations can thus be advanced for the fact that, following a given dose of β-carotene, a rat with hyperthyroidism accumulates more vitamin A than a normal rat (Johnson & Baumann, 1947): either (i) because of improved absorption the β-carotene is presented more efficiently to the enzyme system or (ii) the thyroid hormone acts directly on the enzyme system. According to Rosenberg & Sobel (1953) the alloxan-diabetic rat exhibits impaired capacity to convert β-carotene into vitamin A in isolated intestinal mucosae.

There is, of course, no doubt that the efficiency of β-carotene absorption from food-stuffs is very variable (cf. Hume & Krebs, 1949); this is not surprising when it is recalled that carotene is often present in non-fatty foods which are incompletely broken down in the alimentary tract. It is important to note, however, that mineral oils such as liquid paraffin seriously reduce carotene absorption (Burns, Hauge & Quackenbush, 1951).

One of the most intriguing problems in the natural history of vitamin A is the biogenesis of the vast stores of preformed vitamin found in fishes such as halibut, sturgeon, tunny and soupfin shark and in mammals such as whales. The picture of a food chain from the β-carotene of phytoplankton through the zooplankton to fishes is incomplete and inadequate, but Kon and his colleagues (Kon, 1954) have shown that quite large quantities of vitamin A occur in marine invertebrates such as euphausids and decapods, concentrated almost entirely in the eyes.

This work fills a gap in the food chain from phytoplankton synthesizing carotenoids to fish livers rich in vitamin A. There may well be sufficient β-carotene in the food of young euphausids to account for the vitamin A which they accumulate, but it is not clear how the older euphausids feeding on zooplankton continue to accumulate vitamin A. The principal carotenoid of zooplankton is astaxanthin which is not regarded as a vitamin A precursor. The idea that certain organisms may be able to utilize astaxanthin for the formation of vitamin A has attractions, but there is no convincing evidence in its favour.

4. Vitamin A and Vision

Vitamin A is concerned with both photopic and scotopic vision. The photopic luminosity curve ($\lambda_{max.}$ 560 mμ.) closely resembles the absorption curve for iodopsin, a pigment obtained from the all-cone retina of the fowl (see Hartridge, 1950; Granit, 1947). Electrophysiological studies reveal, in addition to the broad luminosity curve, responses confined to narrow spectral regions (Granit, 1947), and similar phenomena are recorded by other methods (Hartridge, 1950; Motokawa, 1949; Wright, 1943). There is, however, no generally accepted physiological theory of colour vision, but it seems likely that the chemical processes occurring in photopic vision are fundamentally the same as those operating in scotopic vision where either vitamin A_1 or vitamin A_2 is the key substance.

The scotopic luminosity curve with $\lambda_{max.}$ 500 mμ. (or $\lambda_{max.}$ 522 mμ. for species in which vitamin A_2 predominates over vitamin A_1) corresponds with the absorption spectrum of rhodopsin (or porphyropsin). If isolated retinas, dissected out in a dim red light, are gently shaken in normal saline, the outer segments of the rods are broken off; they are free from nuclei, and remain suspended in the medium after filtration through wire gauze. If a solution of sucrose (0.88 M) containing the rod outer segments is centrifuged, practically all other retinal fragments are sedimented (Saito, 1938; Bliss, 1948; Collins & Morton, 1950a; Collins, Love & Morton, 1952a).

The structure of the rod outer segment (guinea-pig eye) has been revealed by electron microscopy (Sjöstrand, 1949) to consist of a stack of some 2,000 discs ($d = 2.1$ μ., thickness 3 mμ.). Chemical analysis (Collins, Love & Morton, 1952b) indicates the absence of deoxypentosenucleic acid and the presence of a good deal of phospholipid. The rod outer segments owe their pink colour to the light-sensitive chromoprotein rhodopsin which is efficiently extracted in a dim red light by a 1% aqueous solution of digitonin (acting as a detergent). The absorption spectrum of rhodopsin is shown in fig. 3. The peak at 270 mμ. is due to the tyrosine and tryptophan in the protein moiety which Wald calls opsin. The lower the ratio of intensity of absorption at 270 mμ. to that at 500 mμ., the smaller the amount of extraneous protein and the " purer " the rhodopsin preparation.

The bleaching of rhodopsin by light was studied by Schneider, Goodeve & Lythgoe (1939) and Goodeve, Lythgoe & Schneider (1942) using the notation :

γ = quantum efficiency, number of chromophores reacting after absorption of one quantum of monochromatic light (within the region covered by the broad absorption band) ;

α = molar extinction coefficient (one chromophore assumed per molecule).

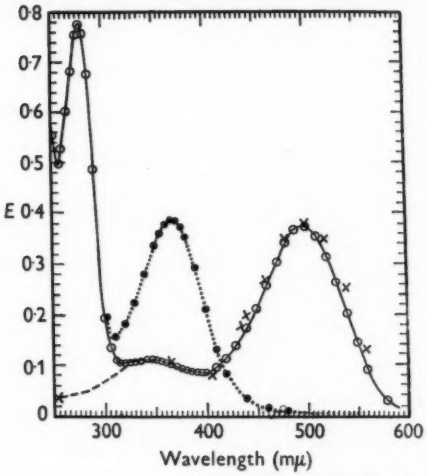
They found $\alpha\gamma = 24{,}000$ at $\lambda_{max.}$ 500 mμ. and, by determining $\alpha\gamma$ at various wavelengths, they measured indirectly the absorption spectrum of the rhodopsin chromophore (see fig. 3).

Wald (1934, 1935–36) had earlier shown that freshly bleached whole retinas yielded to chloroform the substances retinene$_1$ or retinene$_2$, the former being characteristic of mammalian eyes and the latter being restricted largely to those of freshwater fishes and a few amphibians (see Table III).

TABLE III. PROPERTIES OF ALL-*TRANS* RETINENES

Solvent	Retinene$_1$		Retinene$_2$	
	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$
Ethanol . .	383	42,900	400	59,500
$SbCl_3$ reagent	664	108,000	735	112,800

FIG. 3. ABSORPTION SPECTRUM OF RHODOPSIN



Collins, Love & Morton (1952a) by permission of Biochemical Society

Abscissae: wavelength (mμ.)
Ordinates: *E*

E (extinction, log I_0/I) read directly using 1 cm. cell

o——o absorption spectrum of a very pure solution of cattle rhodopsin, pH 9.2

× × × × relative photochemical efficiencies $[(\varepsilon_\lambda \times \gamma_\lambda)/\varepsilon_{500\ m\mu.} \times \gamma_{500\ m\mu.}$ multiplied by a factor so that the two curves correspond at 500 mμ.; after Schneider, Goodeve & Lythgoe, 1939]

• . . . . • absorption spectrum of bleached solution (cf. Collins & Morton, 1950a)

- - - - - - extrapolation indicating probable absorption of the rhodopsin chromophore below 320 mμ.

Lythgoe (1937), on the other hand, had shown that after extracted rhodopsin had been destroyed by light, the solution displayed absorption spectra varying with pH; he described the absorbing entity as "indicator yellow" ($\lambda_{max.}$ 440 mμ. in acid, 365 mμ. in alkali). Lythgoe & Quilliam (1938) had recorded an intermediate state ($\lambda_{max.}$ 470 mμ.) with photochemical bleaching at 5° C.; this they ascribed to "transient orange".

Collins & Morton (1950b) cooled rhodopsin solutions to −70° C. and then exposed the solid to bright light. If the material was allowed to come to room temperature in the dark, half the original pigment was regenerated (although $\lambda_{max.}$ was slightly displaced) together with half the maximum yield of indicator yellow. The process was repeated, each time with a halving of the rhodopsin absorption but with no further displacement of $\lambda_{max.}$ (see fig. 4). The altered regenerated pigment was designated *iso*rhodopsin. Wald, Durell & St George (1950), by measurements at −15° C. and −45° C., found evidence for the presence of *lumi*rhodopsin and *meta*rhodopsin as well as *iso*rhodopsin.

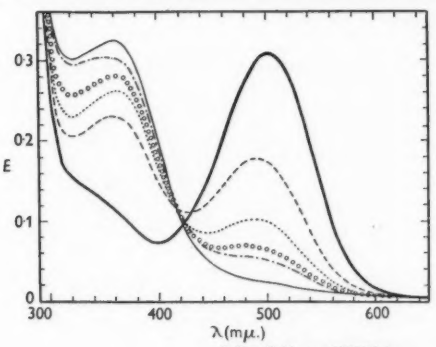
Transient orange is a mixture of these intermediates, but the structural differences have still to be established. Wald & Brown (1952) have made the interesting observation that sulphydryl groups are formed, apparently at least two and possibly three SH groups for each retinene molecule being set free during the bleaching of rhodopsin.

The fact, that if bleached retinas are left to stand for an hour or so vitamin A and not retinene can be extracted by chloroform (Wald, 1935–36), is attributed to alcohol dehydrogenase working in conjunction with coenzyme I (diphosphopyridine nucleotide, DPN) (Wald & Hubbard, 1949 ; Bliss, 1949 ; Wald, 1951).

The ready preparation of retinenes led Ball, Collins, Morton & Stubbs (1948) and Ball, Collins, Dalvi & Morton (1949) to show that they would combine with aliphatic amino compounds to give indicator yellow analogues.

Retinene$_1$ combines with methylamine to form a yellow crystalline substance $C_{19}H_{27}.CH{=}N{-}CH_3$ (retinylidinemethylamine) which has the chromophoric properties of indicator yellow (Pitt, Collins, Morton & Stok, 1955). From the work of Collins (1953) it appears that indicator yellow is formed from rhodopsin rather than from retinene; thus, by adding formaldehyde to rhodopsin at pH 9, the amino groups are blocked, but on irradiation indicator yellow is still formed despite the absence of the free amino groups which would be required if retinene were the precursor of indicator yellow.

FIG. 4. REGENERATION OF RHODOPSIN



Collins & Morton (1950b) by permission of Biochemical Society

Abscissae: wavelength (mμ.)
Ordinates : *E*

——— curve representing fresh unbleached frog rhodopsin solution

- - - - - - curve obtained after "irradiating" solution at − 70° C. and allowing it to reach equilibrium in the dark at room temperature

. . . . . } curves showing effect of repeating above process 3 times
o o o o o
. . . . . }

——— curve obtained after complete bleaching at room temperature

Note that the $\lambda_{max.}$ for rhodopsin differs from that for the regenerated product and that the initial curve does not pass through the approximately isosbestic* point.

* An isosbestic point occurs at a wavelength at which the molecular extinction coefficients of the two separate components of a simple absorbing mixture are the same. When, as in fig. 4, the components are interconvertible, the isosbestic point is the fixed wavelength at which the curves intersect, i.e., 415 mμ.

The regeneration of rhodopsin after photochemical bleaching is a problem of great physiological and chemical interest, but the present position does not lend itself to brief exposition (see Collins, 1954; Wald, 1954b).

If it is accepted that vitamin A alcohol is delivered to the retina, retinene is thereupon formed by DPN and alcohol dehydrogenase, and apparently it retains the stereochemical structure (*cis-trans*) of the parent vitamin. Wald considers that the vitamin A of fish-liver oils is rich in a precursor of "active" retinene (neoretinene *b*), i.e. a vitamin A isomer is present, distinct from all-*trans* vitamin A or neovitamin A. He finds, however, that the retinene formed when rhodopsin is bleached is the all-*trans* isomer which must be isomerized (? enzymically) to "neoretinene *b*" before it can recombine with opsin to form the normal rhodopsin. The existence of a retinene isomerase (Hubbard, 1955) accounts for the very successful regeneration of rhodopsin using all-*trans* vitamin A recorded by Collins, Green & Morton (1953, 1954).

The task of explaining the absorption spectrum in the visible region of rhodopsin and iodopsin in chemical terms still presents great difficulty inasmuch as no known interaction between a protein and an aldehyde can account for their colours. There is, however, no competitor to retinene as the key substance. The attractive notion that either or both of the substances, rhodopsin and transient orange, are free radicals is not supported by measurements of paramagnetic resonance (Pitt & Tinkham, 1955).

Advances in knowledge concerning visual pigments have often sprung from improved experimentation, and technical developments in electrophysiology, spectroscopy and biochemistry have borne much fruit. Shrewd speculation has also sometimes set new investigations going. At present the factual evidence cannot all be fitted into a neat framework, and a new picture will inevitably be more complicated. Thus the question as to how many pigments must be recognized is by no means easy to answer, and the more numerous they are the more difficult it becomes to account for them in chemical terms. There is an important difference, for example, between Dartnall and Wald over the absorption spectrum of porphyropsin: the former gives $\lambda_{max.}$ as 533 mμ., and the latter as 522 mμ. (see Table IV). This discrepancy leads to very different interpretations concerning the retinal pigment of *Xenopus laevis* ($\lambda_{max.}$ 519 mμ.); Dartnall regards this as a new and distinct pigment, while Wald thinks it is mainly porphyropsin. The status of Dartnall's 467 mμ. pigment of tench (*Tinca tinca*) and his 510 mμ. pigment of bleak (*Alburnus lucidus*) would be higher if the vitamin concerned (A_1 or A_2) were known (Table IV). Euphausiopsin from the euphausid retina is another claimant for recognition (Kampa, 1955).

Dartnall has shown that, when spectral sensitivity curves corresponding with visual pigments are plotted on a frequency scale (rather than a wavelength scale), they have practically the same shape but differ of course in position. The bodily displacement of broad absorption curves might be expected to have a simple explanation in terms of chromophoric theory but this has so far eluded notice.

It is now possible by direct observation and by photoelectric recording to measure the amount of rhodopsin in the living eye (Rushton, 1953; Rushton & Campbell, 1954; Campbell & Rushton, 1954; Rushton, Campbell, Hagins & Brindley, 1955), and from this and other evidence (Willmer, 1955) it is now clear that purely photochemical reactions play a minor part in light-adaptation. This had been sensed by Lythgoe (1940) and by Craik & Vernon (1941), and Wald (1954b) saw "no escape from the conclusion that a high degree of light adaptation can be achieved with very little bleaching of rhodopsin". It is nevertheless clear that a rod can be excited by a single quantum of light and that a few rods (2–8) so excited can be translated into vision.

The electrophysiology of the mechanism which links the absorption of light by a retinal pigment to a message received

TABLE IV. RETINAL PIGMENTS

Designation	Reference	$\lambda_{max.}$ (approx.) (mμ.)	Vitamin concerned	Source
Rhodopsin (visual purple)	cf. Collins & Morton (1950a), Wald & Brown (1953)	500	A_1	Frog retinas, mammalian retinas
Cephalopsin*	Bliss (1948), St George & Wald (1949)	490	A_1	Squid (*Loligo peali*)
Porphyropsin (visual violet)	cf. Wald (1953)	522 (533)†	A_2	Freshwater fish retinas, tench retinas, pike (*Esox lucius*) retinas
Iodopsin	cf. Wald (1953), Wald, Brown & Smith (1955)	555–562	A_1	Rod-free retinas, including fowl retinas
Cyanopsin‡	Wald, Brown & Smith (1953)	620	A_2	In-vitro action of retinene$_2$ and protein from fowl retinas
Euphausiopsin	Kampa (1955)	462	A_1	Shrimp (*Euphausia pacifica*)
Violet receptor	Auerbach & Wald (1954)	436	?A_1	Human
Pigment 467	Dartnall (1952a)	467	?A_1 or A_2	Tench (*Tinca tinca*) retinas
Pigment 470	—	470	—	Living guinea-pig eye§
Pigment 519	Dartnall (1954), see also Wald (1955)	519	A_2	Clawed toad (*Xenopus laevis*)
Pigment 570	Denton & Pirenne (1954)	560–570	?A_1 or A_2	Clawed toad (*Xenopus laevis*)
Pigment 510	Dartnall (1952b)	510	?A_1 or A_2	Bleak (*Alburnus lucidus*)
Pigment 510	—	510	A_1	Living guinea-pig eye§
Pigment 535	—	535	A_1	Living grey-squirrel eye‖

* Regarded by Bliss (1948) and by St George & Wald (1949) as rhodopsin.
† See Dartnall (1952a).
‡ See Granit (1941) for electrophysiological evidence.
§ See Weale (1955a).
‖ See Weale (1955b).

by the brain is complex indeed, but the association between vitamin A deficiency and defective low intensity vision has not been questioned.

5. Systemic Action

The systemic mode of action of vitamin A—the biochemistry of its anti-xerophthalmic and growth-promoting actions—is still but little understood. A beginning has been made in the discovery that certain aspects of sterol metabolism are deeply deranged in the rat suffering from avitaminosis A (Lowe, Morton & Harrison, 1953; Heaton, Lowe & Morton, 1955; Morton, 1955). There is no doubt that the compound which the above authors designate "SC" (absorption maxima at 232 mμ., 276 mμ., 285 mμ. and 317 mμ. for the acetate) is present to a vastly greater extent in the livers of rats suffering from avitaminosis A as compared with normal animals. In other tissues the relative proportions of 7-dehydrocholesterol and cholesterol undergo marked changes. How far the results with rats also apply to other species at different stages in the life-cycle is now being studied.

In earlier work on water-soluble vitamins, clues to modes of action came from detecting accumulations of metabolites in deficiency states. Although knowledge about the vitamin A deficiency syndrome is in this sense lagging behind, the work on SC in the rat may be a beginning of new developments.

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VITAMIN E

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1 Chemistry of the different forms of tocopherol
2 Vitamin E activity of certain redox dyes
3 Vitamin E in anoxia and hyperoxia
4 Vitamin E in the premature infant
5 Vitamin E in human dietetics
References

Vitamin E was discovered more than thirty years ago as a factor necessary to prevent a particular form of reproductive failure in female rats, which was characterized by the death and resorption of the foetuses. Hence arose the description "reproductive vitamin", and later the name "tocopherol", from the Greek for "child-bearing". The progress of research, however, has indicated a scope of activity for vitamin E which seems to be continually widening, as more knowledge is gained. Thus it was soon found that the male reproductive processes were affected by its deficiency even more than the female, with irreparable damage to the testes. Deficiency of the vitamin was next found to be the prime cause of "nutritional muscular dystrophy" in guinea-pigs, rabbits and rats, which extended the field of influence beyond the ambit of sex. In virgin female rats a peculiar brown pigmentation of the uterus had previously been noticed, which was now recognized as a special form of dystrophy, with the appearance of pigment granules similar to those sometimes seen in the senile human heart. Another lesion consistently observed in the rat was tubular degeneration of the kidneys. The normal brown pigment was lost from the anterior surfaces of the incisor teeth. Deficiency in chicks caused a tendency to oedema, termed "exudative diathesis", and encephalomalacia.

Recognition of the chemical properties of vitamin E as an antioxidant coincided with progress in other directions. Thus, in rats, deficiency of vitamin E caused defective storage of the readily oxidizable vitamin A. In rats, chicks or pigs given excess of cod-liver oil, vitamin E prevented brown discoloration of the fat deposits, which otherwise resulted from the oxidation of highly unsaturated fatty acids derived from the oil. Another role for vitamin E was found in protein deficiency. In rats deprived of both protein and the vitamin, death ensued from massive necrosis of the liver, but the necrosis could be prevented by tocopherol, cystine or an unknown factor present in certain strains of yeast. A further highly important development was the discovery that tocopherol may influence the liability of the red blood corpuscles to haemolysis. This influence could be conveniently demonstrated in vitro on rats' erythrocytes, with dialuric acid as a haemolysing agent.

The effects of vitamin E deficiency, therefore, present in themselves a bewildering series of pictures, which vary in their combinations according to the species, age and diet of the victim. Further perplexing complications, however, have been introduced by claims that substances quite different from the vitamin, notably certain redox dyes and other substances with reducing or antioxidant activity, may fulfil at least some of its biological functions. There certainly seems no doubt that such substitutions can occur, but the evidence is conflicting as regards the range of substances which possess this activity, and the particular injuries which may be prevented or cured. An impression of the complexity of the many interdependent problems which have arisen in this field may be gained from a recent review by Dam (1955).

It is obvious that a full discussion of modern knowledge of vitamin E would be beyond the scope of a short article, even if one felt competent to undertake such a task. Attention, therefore, will be limited to certain selected topics. These may provide clues for use in future comprehensive studies on the mode of action of the vitamin, and on its practical importance in nutrition.

1. Chemistry of the Different Forms of Tocopherol

It has long been known that vitamin E occurs in its natural sources as a series of closely related compounds which differ in the number and position of the methyl groups attached to the chromane nucleus (see fig. 1). In the forms of tocopherol which are already well known, designated α, β, γ, δ, these differences in methylation have been shown to affect profoundly the biological activity, antioxidant power in vitro, strength of adsorption in chromatography, and the ability to give coloured compounds with diazotized *o*-dianisidine. Thus the tocopherols mentioned may be arranged in the order $\alpha > \beta \geqslant \gamma > \delta$ according to biological activity; $\delta > \gamma \geqslant \beta > \alpha$ according to antioxidant power; or $\alpha > \beta = \gamma > \delta$ according to the strength of adsorption. Only γ- and

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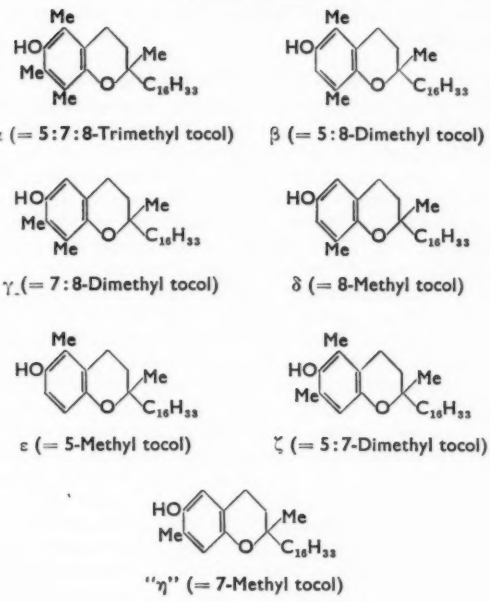
FIG. 1. CHEMICAL STRUCTURE OF THE TOCOPHEROLS


α (= 5:7:8-Trimethyl tocol)
β (= 5:8-Dimethyl tocol)
γ (= 7:8-Dimethyl tocol)
δ (= 8-Methyl tocol)
ε (= 5-Methyl tocol)
ζ (= 5:7-Dimethyl tocol)
"η" (= 7-Methyl tocol)

δ-tocopherols react with *o*-dianisidine. The apparent anomaly between biological activity and antioxidant power, as seen, for example, between α- and δ-tocopherols, may be explained by differences in the ease of transfer into animal tissues. Thus α-tocopherol passes into the tissues much more readily than the other forms.

During the past three years, considerable advances have taken place in the technique for estimation of the tocopherols, which have resulted in the recognition by British workers of two naturally occurring tocopherols previously only known as products of chemical synthesis.

Brown (1952a, 1952b) devised a method for separating the tocopherols on vaseline-coated filter-paper with 75% ethanol as the mobile phase. Eggitt & Ward (1953) made this method quantitative by substituting liquid medicinal paraffin for the vaseline and by carrying out the chromatography in an atmosphere of nitrogen. When examining wheat-germ oil, Brown reported that besides the expected spots due to α- and β-tocopherols, which had R_F values of 0.5 and 0.72, respectively, another spot was present with an R_F value of 0.93. This spot reduced ferric chloride, but did not couple with diazotized *o*-dianisidine. It was, therefore, not δ-tocopherol which he reported as having a closely similar R_F of 0.84. Re-examination of the new spot by Eggitt gave, under modified conditions, an R_F value of 0.68 as compared with 0.65 for δ-tocopherol. On paraffin-coated filter-paper, increasing the total number of methyl groups in the chromane nucleus (see fig. 1) decreases the R_F value stepwise. Thus β-tocopherol runs to the same position as its isomer, γ-tocopherol. This suggested that a spot running close to that of δ-tocopherol would probably be an isomer. Examination of the spectra of the nitroso derivative, and the fact that it did not couple with diazotized *o*-dianisidine, strongly suggested that the new spot was 5-methyl tocol, to which Eggitt gave the prefix ε.

Confirmation of the identity of this spot was provided by Green, Marcinkiewicz & Watt (1955), who also detected yet another tocopherol. These workers devised a two-dimensional method for paper chromatographic separation of the tocopherols. The first dimension consisted of adsorption of the tocopherols on zinc carbonate coated filter-paper with *cyclo*hexane as the developing solvent. This separated the tocopherols into groups not according to the total number of methyl groups, but according to the number adjacent to the hydroxy group (again see fig. 1). Thus α- has two such hydroxyls, β-, γ- and ε- have one and δ- has none, with R_F decreasing accordingly. The second dimension was one of partition chromatography as employed by Eggitt. This enables the separation of ε- from β- and γ-tocopherols. When this method was applied to bran oil, besides the spots due to α-, β- and ε-tocopherols, a fourth spot appeared which ran with α- in the first dimension and β- in the second. This spot did not couple with diazotized *o*-dianisidine nor did it form a nitroso derivative. This was further evidence that the substance was 5:7-dimethyl tocol, which had already been indicated by its position in the two-dimensional chromatogram. The prefix ζ was given, being the next letter in the Greek alphabet. ε-Tocopherol is the main tocopherol in bran oil, and ζ-tocopherol the main one in barley oil.

The biological potency of these new tocopherols has still to be estimated. Jacob, Sutcliffe & Todd (1940) found that synthetic ζ-tocopherol was as active as α-tocopherol by the rat resorption test, and from its structure it would appear that ε-tocopherol would have an activity near to that of β-tocopherol.

Work is proceeding in an attempt to find the only other possible methylated tocol, 7-monomethyl tocol, which will presumably be called η-tocopherol (Green, personal communication, 1955).

My thanks are due to R. J. Ward, B.Sc., for help with this section.

2. Vitamin E Activity of Certain Redox Dyes

The first reports of the vitamin E activity of methylene blue came from Dam and his colleagues, and have been discussed in his recent review (Dam, 1955). At Cambridge, Moore, Sharman & Ward (1953a, 1953b, 1953c, 1954) have failed, under somewhat different experimental conditions, to confirm Dam's most striking finding—that vitamin E can prevent resorption gestations in rats deficient in vitamin E—and they have had no success in preventing degeneration of the testes. They found clear evidence, however, of the potency of the dye in completely and permanently preventing "brown uterus", which is a lesion only known to occur in deficiency of vitamin E. Kidney degeneration was also prevented. Their complete findings were as follows:

Full protection by methylene blue against	*No protection by methylene blue against*	*Slight protection by methylene blue against*
Brown uterus	Resorption gestations	Dental depigmentation
Kidney degeneration	Degeneration of testes	
Defective storage of vitamin A	Haemolysis	

It appears, therefore, that methylene blue has undoubtedly some degree of vitamin E activity, which can be observed without difficulty in the rat uterus. The diversity of findings on protection against resorption gestations suggests that special circumstances may be necessary for the demonstration

of activity. Thus Markees (1953) considers that methylene blue may act by protecting tocopherol, thus prolonging the period of dietary restriction during which litters can still be produced.

Moore and his colleagues found that certain other redox dyes, or similar substances, including thiodiphenylamine, Bindschedler's green, methyl violet, rosaniline, and malachite green, shared the activity of methylene blue. Certain other redox dyes, however, were inactive against brown uterus. The same failure was observed with various other substances, including ascorbic acid, cystine, Antabuse and nordihydroguaiaretic acid, which had been found in Dam's laboratory to exert vitamin E activity, in one way or another.

In attempting to explain why some redox dyes should have partial vitamin E activity we may recall the results of two types of biochemical study. Firstly it has been shown by Dam's colleagues, Glavind & Hartmann (1949a, 1949b, 1949c), that the leuco forms of redox dyes may be oxidized to the coloured compounds by fatty acids in the presence of haemin. Tappel (1953, 1954) and Maier & Tappel (1955) made experiments on this reaction in the reverse direction, and demonstrated that redox dyes could retard the oxidation of unsaturated fat by oxygen in the presence of haematin. These observations suggest a form of mechanism to account for Dam's finding that methylene blue can prevent the brown discoloration of the body fat otherwise induced by cod-liver oil. It is puzzling, however, to explain why peroxidized fats can react not only with leuco forms of biologically active dyes, such as malachite green, but also with the leuco form of 2:6-dichlorophenolindophenol, in which Moore, Sharman and Ward could detect no vitamin E activity.

The second type of biochemical study, apparently originated by Ehrlich (1885), has concerned the ability of redox dyes to take part in enzyme systems. A review of all the numerous instances in which methylene blue has been used in enzyme research as a hydrogen acceptor would be out of place here, but it appears that the effect of the dye is usually to increase the rate of oxygen uptake. Thus Barron & Harrop (1928) found that in mammalian erythrocytes oxygen uptake and glycolysis were greatly accelerated by the presence of methylene blue. Barron (1929) made similar studies on the eggs of sea-urchins and star-fishes, and observed the same stimulation. At first sight it is surprising, therefore, that vitamin E, which can be partially replaced by methylene blue, has a retarding effect on respiration, at least when the measurements are made on isolated tissues. Thus numerous workers have shown that when rabbits, rats or hamsters are deprived of vitamin E the oxygen uptake of their muscles in vitro is much increased (Victor, 1934; Madsen, 1936; Telford, Emerson & Evans, 1936; Friedmann & Mattill, 1940; Biddulph & Meyer, 1942; Houchin & Mattill, 1942; Kaunitz & Pappenheimer, 1942; Hummel & Basinski, 1948).

It seems an attractive hypothesis that in avitaminosis E the products of intermediary metabolism become abnormal either in quality or in quantity, and that methylene blue may help by speeding up the removal of such products. If it could be proved that tocopherol and methylene blue intervene in metabolism at different points, and if the anatomical injuries incurred could be shown to depend on the extent of the biochemical failure, then the partial vitamin E activity of the dye could readily be explained. Speculation on the biochemical systems involved, however, is made difficult by our lack of detailed knowledge of the interplay between the aqueous and fatty phases. Thus in artificial enzyme systems methylene blue presumably acts in the aqueous phase, whereas in studies of antioxidant power tocopherol is usually dissolved in fat.

3. Vitamin E in Anoxia and Hyperoxia

As our next topic we shall take the role of vitamin E in enabling the intact animal to adjust itself to inadequate or excessive supplies of oxygen. Evidence of this role has been found in observations of the increased sensitivity to changes in the oxygen supply of animals deficient in vitamin E. Thus Hove, Hickman & Harris (1945) found that in rats subjected to reduced atmospheric pressures the average survival time was nearly 90% longer in animals dosed with tocopherols than in deficient animals. In guinea-pigs, but not in rats, the survival time was greatly lengthened by injections of α-tocopheryl phosphate. Telford, Wiswell & Smith (1954), working with rabbits, confirmed the protective action of tocopherol, when given orally.

It might seem tempting to link the decreased resistance to anoxia caused by deprivation of vitamin E with the increased oxygen demands of the tissues, as demonstrated on muscle in vitro. Experiments in Aberdeen by Taylor (1953), however, have indicated that deficiency of vitamin E also reduces the resistance to excess of oxygen. In this work rats were brought up, from weaning to an age of nine months, on a diet deficient in vitamin E, which was supplemented in some animals by adequate doses of tocopherol. They were then exposed, at 5 atm. pressure, to oxygen (98% O_2) for a standard period of 50 min. For groups of 14 animals the following results were obtained:

	Developed convulsions	*Died*
Group A (10 mg. tocopherol weekly)	4	1
Group B (no tocopherol)	14	13

There were indications, at least, that tocopherol decreased the damage to the lungs caused by excess of oxygen. Tocopherol was also beneficial against haemolysis, which was later detected as a further effect of the hyperoxia (Taylor, personal communication, 1955).

These experiments can leave little doubt as to the fact that vitamin E deficiency causes decreased resistance in animals to changes in oxygen. It still remains uncertain, however, whether avitaminosis E should be considered more important than various other dietary factors in decreasing resistance. Thus Hove *et al.* (1945) noticed that the time of survival of rats in anoxia was increased by the removal of fat from their diet.

In view of the partial vitamin E activity of redox dyes it is interesting that methylene blue has been used in human medicine for combating anoxia. According to Dunlop, Davidson & McNee (1949), anoxia in lobar pneumonia or anaemia may be treated by giving oxygen and administering the dye, either orally or by injection. Methylene blue has also been recommended as an antidote to poisoning by coal-gas or by hydrogen cyanide (Brooks, 1932, 1933) and for controlling methaemoglobinaemia (Wendel, 1939).

4. Vitamin E in the Premature Infant

As far as can be judged from the examination of blood specimens, the vitamin E status of normal human infants tends

to be much lower than in adults. In chemical estimations of tocopherol in blood plasma Straumford & Quaife (1946) found an average of 0.34 mg./100 ml. for a series of 56 infants, as against 1.70 mg. for their mothers. In haemolysis tests György, Cogan & Rose (1952) observed that the erythrocytes of new-born infants were much more sensitive to hydrogen peroxide than those of adults, and that this undue sensitivity could be corrected by dosing with tocopherol.

Regarding the premature infants we may recall that Owens & Owens (1949) suggested that deficiency of vitamin E might be a primary factor in producing retrolental fibroplasia, a cause of permanent blindness. In support of this possibility Callison & Orent-Keiles (1951) observed eye lesions, resembling retrolental fibroplasia in their symptomatology, in weanlings reared by mother rats partially deficient in vitamin E. The discovery of the sensitivity to haemolysis in rats deficient in vitamin E must further have suggested to many workers that the incidence of kernicterus, which is associated with haemolysis in the premature infant, might also be influenced by the vitamin E status. In applying the ordinary dialuric acid test to the erythrocytes of premature infants Cohen, DeMetry & Herrmann (1951) failed to observe haemolysis, but, in studies of the sensitivity of erythrocytes to hydrogen peroxide, Gordon & DeMetry (1952) and Mackenzie (1954) made extensive observations on premature infants with results which paralleled those of György *et al.* (1952) on normal infants. Thus it was abundantly proved that the erythrocytes were unduly sensitive to haemolysis; it was also demonstrated that this increased sensitivity could be reduced by dosing with tocopherol.

Our attention must next be turned to the important observations of the London workers, Ashton, Ward & Serpell (1953). Without reference to the vitamin E status these workers exposed young kittens for several days to an atmosphere containing 60–80% of oxygen. This treatment was found to have a profound effect on retinal vascularization, with obliteration of the ingrowing retinal complexes. After restoration of air, moreover, the normal architecture of the retina was not restored. The abnormalities included the invasion of the vitreous by blood vessels and retinal detachment, and were considered relevant to the genesis of retrolental fibroplasia in man.

From our preceding section we may remember that Taylor (1953; and personal communication, 1955) has found that the survival of rats exposed to excess of oxygen, and their resistance to haemolysis, is profoundly influenced by their vitamin E status. We may look forward with interest, therefore, to further evidence of a possible combination between excess of oxygen and inadequacy of vitamin E as an important factor for precipitating metabolic catastrophes in premature infants.

However, Cohen *et al.* (1951) have pointed out that, besides those already mentioned, certain other factors, including the administration of iron and of water-miscible solutions of fat-soluble vitamins, and the use of certain detergents, have been under suspicion as possible causes of retrolental fibroplasia. The recent observations of the Oxford worker, Allison (1955), have certainly justified such suspicions. The occurrence of kernicterus and haemolytic anaemia in a small series of premature infants, with no evidence of Rh incompatibility, could be explained only by grossly excessive dosing with the water-soluble vitamin K analogue Synkavit (tetrasodium-2-methyl-1:4-naphthohydroquinone diphosphate). In Derby the death by kernicterus of six babies was attributed by Laurance (1955) to the same cause. Moore & Sharman (1955) have collaborated with Allison in testing the effect of injections of Synkavit on rats. Little or no effect was seen in normally nourished animals. In deficiency of vitamin E, however, the injections were followed by haemolysis and by a profuse haemoglobinuria. Certain other vitamin K substitutes were even more toxic than Synkavit, but others were much less toxic. No injury could be detected after massive injections of vitamin K_1.

It seems that in the rat haemolysis may be promoted by hyperoxia or by the injection of certain chemicals. Protection against these stresses is given by vitamin E. These findings deserve consideration for application in the management of premature human infants. But the experience with Synkavit would emphasize the need for caution before the institution of massive therapy, even with vitamin E.

5. Vitamin E in Human Dietetics

In contrast to the profusion of our knowledge of the ill effects of vitamin E deficiency in experimental animals, we have still very little evidence on which the importance of the vitamin for human nutrition can be appraised. Attempts to correlate habitual abortion with vitamin E deficiency seemed promising at one period. The current view, however, is that normal deliveries follow spontaneously after abortion so often that it is difficult to assess statistically the effect of any form of treatment. The treatment of muscular dystrophies, similar to those observed in experimental deficiencies, has been disappointing. We may remember, however, that the paralysis associated with chronic vitamin E deficiency in the rat obstinately resists cure by tocopherol therapy, or by any other known means. The observation of normal ranges for plasma tocopherol in cases of human muscular dystrophy may, therefore, seem more cogent as an argument against vitamin E deficiency being implicated.

Shute (1949) and his colleagues have published an extensive series of papers, largely in a special journal, on the treatment of common human diseases, mainly cardiac and vascular disorders, by very heavy doses of tocopherols. This enthusiastic worker has expressed impatience with controlled clinical trials, and his claims, although strongly supported in some quarters, have been freely criticized in others. Several authorities have failed to confirm that tocopherol confers any benefit in the treatment of heart or vascular diseases. Certain claims, however, appear to justify further investigation because they have already satisfied certain statistical standards. Thus in Britain α-tocopherol was found to give significant benefit in intermittent claudication by Ratcliffe (1949) in Manchester, and in the healing of certain forms of leg ulcers by Lee (1953) in London. It seems to me that treatment with tocopherol may indeed be beneficial in some patients. It may be extremely difficult, however, to appreciate the reality of the benefit in individual cases, either when the disease tends to improve spontaneously or when a complete cure is impossible. Thus in Lee's investigation the ulcers were usually healed by conservative treatment whether tocopherol was given or not, and its beneficial action had to be decided from the speed of the cure. On the other hand Ratcliffe's patients could not be completely cured of their claudication, and the benefit of tocopherol had to be deduced from the

numbers of patients who could walk a certain distance, without pain, with and without therapy.

Lack of definite proof of the occurrence of avitaminosis E in the adult human, or of clear responses of vascular diseases to massive therapy, must not, however, lead us to ignore the importance of tocopherol as a dietary factor. Even if we assume that deficiency of vitamin E never occurs, it seems reasonable to infer that this freedom from deficiency is associated with the presence of large amounts of tocopherol carried in the plasma. It is perhaps insufficiently realized that the concentration by weight of vitamin E in the plasma exceeds that of vitamin A about 30-fold, and that there is an even greater disparity compared with the almost immeasurably small traces of vitamin D. From a mixed diet the α-form of tocopherol seems to be taken into the blood in preference to other forms. For each individual a characteristic steady level of tocopherol seems to be maintained in the plasma, with only temporary responses to dietary fluctuations. These circumstances suggest that vitamin E has an essential role to play in the human no less than in experimental animals, and it may be hoped that in due time its significance will be established. Perhaps a start will be made from the observations of the low vitamin E status in infants, and the risks which appear to be entailed thereby, as described in preceding sections. Interesting possibilities are also opened up by the recent well-planned investigation on Welsh hostel students by Butler & McKnight (1955), in which α-tocopherol was found to be beneficial in the treatment of primary dysmenorrhoea.

In the British diet tocopherols are supplied in vegetable oils, including margarine and cooking fats, eggs, dairy products, green vegetables and meat. Flour and bread would be important sources if they were produced without chemical treatment deleterious to the vitamin. Thus Moran, Pace & McDermott (1953) have shown that both of the "improvers", nitrogen trichloride and chlorine dioxide, are highly destructive. Working with Dr L. J. Harris, my colleagues and I have confirmed that chlorine dioxide in concentrations used commercially destroys virtually all the tocopherol in flour of the usual 80% level of extraction.

It was on the basis of the classical experiments on canine hysteria by the late Sir Edward Mellanby (1946), to whom this number of the Bulletin is dedicated, that the use of agene as a flour improver was prohibited in the USA and more recently in Great Britain. Chemical considerations ensure that chlorine dioxide cannot replace agene in forming the methionine sulphoximine which is responsible for canine hysteria. The more recently established destruction of tocopherol, however, incriminates both improvers equally. Since the flour is not to be eaten by the dog but by man, a species in which methionine sulphoximine does not readily induce signs of toxicity, the advantages of chlorine dioxide over agene seem at least highly questionable. One feels that Mellanby's distrust for the sophistication of foods might not have been disarmed for long by the banning of agene, and that further chapters on our maltreatment of the "staff of life" have still to be written.

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INTERRELATIONSHIPS OF TRYPTOPHAN, NICOTINIC ACID AND OTHER B VITAMINS

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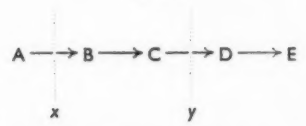
1 B vitamins as coenzymes
2 Tryptophan the precursor of nicotinic acid
3 Pyridoxine, riboflavin and thiamine in tryptophan degradation
4 Vitamin deficiencies as a research tool
References

A major landmark in biochemistry around the turn of the century was the recognition, particularly by Garrod (1909, cf. Garrod, 1923), of the existence of inborn errors of metabolism, in which various congenital pathological states are associated with an inability to carry out specific enzymic reactions. In such cases, the substrate of the inhibited reaction, or derived substances bearing a simple relation to the substrate, are excreted, and much valuable information has thus been obtained on individual steps in metabolic pathways. The number of congenital metabolic errors occurring naturally is small, the majority of mutations which would give rise to such states presumably being lethal or at least highly disadvantageous. Apart from these congenital states there are many acquired states, especially in vitamin deficiencies, in which enzymic reactions are inhibited. The pioneer work of, particularly, Hopkins (cf. Dale, 1948), led to a realization of the importance of vitamins, and a lasting achievement of Sir Edward Mellanby was his co-ordination of academic vitamin research with clinical observations on many pathological states which he showed to be due to nutritional deficiency (cf. Mellanby, 1934).

1. B Vitamins as Coenzymes

It is now well known that by producing a deficiency of a B vitamin one can frequently produce in a laboratory animal an inhibition of enzymic reactions comparable with that in Garrod's inborn errors of metabolism. Many enzymes consist of two parts, a protein constituent, or apoenzyme, conferring specificity, and a prosthetic group, or coenzyme, concerned in the chemical change. In such cases both apoenzyme and coenzyme must be present for reaction to occur. B vitamins form essential parts of many coenzymes. In a B vitamin deficiency the enzymic reactions for which that particular B vitamin forms part of the coenzyme are therefore liable to be inhibited. Thus if we have metabolic transformation of a substance A to a substance E, as shown in fig. 1, then a block at stage *y* (conversion of C to D), due to lack of the appropriate coenzyme, will cause inhibition of formation of D and E and an accumulation of C, and perhaps also of B and A. C is then likely to be excreted in abnormal amount, either unchanged or as simple conjugates or derivatives. Study of the change of pattern of metabolite excretion

FIG. 1



in a B vitamin deficiency may thus give valuable information both on metabolic pathways (by causing excretion of an intermediate not otherwise present in detectable amounts) and on the mode of action of vitamins. Conversely, excretion of an abnormal metabolite after a test dose of a standard substance can be a valuable test for vitamin deficiency, the conversion of tryptophan to xanthurenic acid in pyridoxine deficiency being an example.

B vitamin deficiency states are in general fairly readily attained. A diet based on alcohol-extracted casein, fat mixture (with addition of fat-soluble vitamins), starch, sucrose and a salt mixture is simple to prepare and keeps well. B vitamins are most conveniently given in solution by stomach-tube (cf. Copping, Crowe & Pond, 1951; Dalgliesh, 1955c), appropriate B vitamins being omitted as required. Rats readily tolerate this procedure, and in most cases deficiencies are becoming appreciable by the third or fourth week of the regime. In some cases it is desirable also to inhibit bacterial synthesis of vitamins in the gut, by adding, say, 1% by weight of succinylsulphathiazole to the diet. The substance being investigated can either be mixed in the diet or given by stomach-tube.

2. Tryptophan the Precursor of Nicotinic Acid

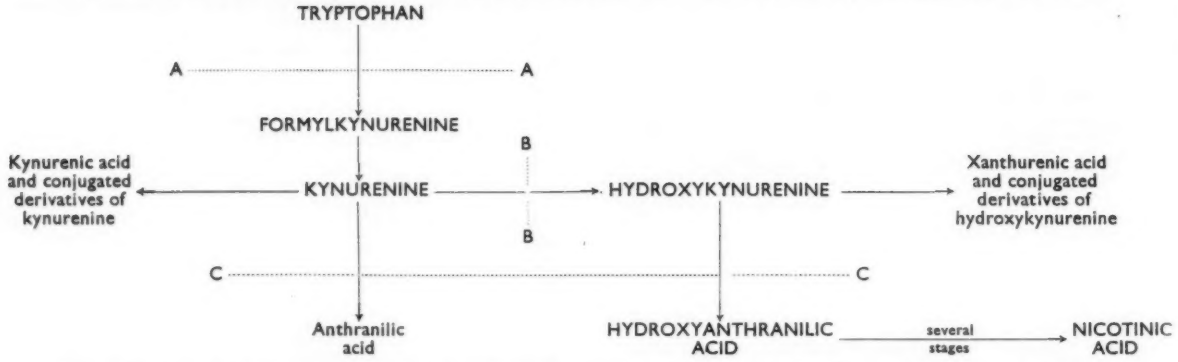
The comparatively recent discovery (Krehl, Teply, Sarma & Elvehjem, 1945) that the essential amino acid, tryptophan, is transformed into nicotinic acid by mammalian tissues was an outstanding event in vitamin research, as B vitamins cannot in general be synthesized by higher animals. (Normally they are derived from the diet or the bacterial flora of the gut.) Formation of nicotinic acid from tryptophan is, moreover, markedly affected by other B vitamins, being inhibited in deficiencies of pyridoxine, riboflavin and thiamine (see, for example, Junqueira & Schweigert, 1948). A brief account is given here of work leading to the correlation of these vitamin interactions by using the techniques outlined above.

In the last 15 years a vast amount of work, reviewed by the author in detail elsewhere (Dalgliesh, 1951, 1955a), has been carried out on tryptophan-nicotinic acid interconversion. The pathway now generally accepted involves successively (neglecting transient intermediates) formylkynurenine, kynurenine, hydroxykynurenine, hydroxyanthranilic acid and nicotinic acid. This pathway is summarized in fig. 2, and the structural formulae of the principal compounds concerned are shown in fig. 3.

3. Pyridoxine, Riboflavin and Thiamine in Tryptophan Degradation

Kynurenic acid and xanthurenic acid, respectively derived from kynurenine and hydroxykynurenine by transamination and cyclization, are by-products of tryptophan metabolism,

FIG. 2. OUTLINE OF PATHWAY FOR CONVERSION OF TRYPTOPHAN TO NICOTINIC ACID

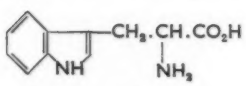


Intermediates on the main pathway are given in capitals, by-products in small type. Pyridoxine participates at stage C . . . C; riboflavin at stage B . . . B; and thiamine at stage A . . . A.

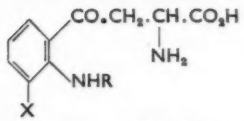
as is anthranilic acid. Both intermediates and by-products are normally excreted in, at most, very small amounts. But administration of tryptophan to pyridoxine-deficient rats results in a large excretion of kynurenine, hydroxykynurenine, kynurenic acid and xanthurenic acid, and of several conjugated derivatives (" detoxication " products) of these substances (Dalgliesh, 1952; cf. Table I). All these metabolites retain the carbon skeleton of the original alanine side-chain of tryptophan, which is known to be lost during nicotinic acid formation. This shows that pyridoxine participates in the reaction in which the side-chain is split off (stage C . . . C in fig. 2), and this is in entire agreement with enzymic investigations which have shown that the appropriate enzyme, kynureninase, requires pyridoxal phosphate as a coenzyme (e.g. Dalgliesh, Knox & Neuberger, 1951; Knox, 1953). On the other hand, in a riboflavin deficiency large amounts of free and conjugated kynurenine and anthranilic acid, and only limited amounts of hydroxylated metabolites, are excreted, showing that a block is occurring at stage B . . . B in fig. 2 (Charconnet-Harding, Dalgliesh & Neuberger, 1953). The technique is particularly valuable in this case, as the appropriate enzyme is so labile that it has never been isolated in a cell-free state, and enzymic techniques are therefore inapplicable. Detailed consideration of the pattern of metabolite excretion moreover suggests that the fundamental reaction is an oxidative phosphorylation, to give hydroxykynurenine phosphate, rather than a simple oxidation (Charconnet-Harding *et al.* 1953).

When, as in the above cases, a block occurs fairly far along a metabolic chain, there is a good chance of its detection. But an absence of metabolite excretion does not necessarily indicate a block at the first stage of a metabolic pathway, most normal pathways being so efficient that little excretion of intermediates in any case occurs. This difficulty can be overcome simply. If a deficiency thought to cause a block at an early stage in a pathway (e.g. at stage *x*, fig. 1) is superimposed on a block known to occur later (e.g. at *y*, fig. 1), then the accumulation of C characteristic of block *y* will cease when block *x* is also imposed, as formation of C is then inhibited. On feeding tryptophan to rats in which a thiamine deficiency has been superimposed on a pyridoxine deficiency, the metabolite-excretory pattern typical of pyridoxine deficiency disappears, but reappears as soon as thiamine is given (cf. Table I). On the other hand, kynurenine given to the doubly deficient animals causes a large metabolite excretion, showing that the block due to thiamine lies between

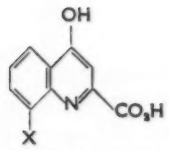
FIG. 3. STRUCTURAL FORMULAE OF PRINCIPAL SUBSTANCES DISCUSSED IN THE TEXT



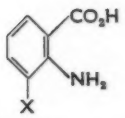
Tryptophan



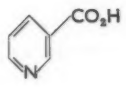
Formylkynurenine (R = CHO ; X = H)
Kynurenine (R = X = H)
3-Hydroxykynurenine (R = H ; X = OH)



Kynurenic acid (X = H)
Xanthurenic acid (X = OH)



Anthranilic acid (X = H)
3-Hydroxyanthranilic acid (X = OH)



Nicotinic acid

TABLE I. EXCRETORY PATTERNS OF TRYPTOPHAN METABOLITES IN SOME DEFICIENCY STATES

Metabolite excreted	Control animals given tryptophan	Pyridoxine-deficient animals given tryptophan	Thiamine-deficient animals given tryptophan	Thiamine-pyridoxine-deficient animals given tryptophan	Thiamine-pyridoxine-deficient animals given kynurenine	Thiamine-pyridoxine-deficient animals given thiamine and tryptophan
Kynurenine	±	+ +	−	±	+ + +	+ +
Kynurenine conjugates	−	+ +	−	−	±	+ +
Hydroxykynurenine	−	+ +	−	±	+ +	+ +
Hydroxykynurenine conjugates	−	+ + +	−	±	+ +	+ + +
Xanthurenic acid	±	+ + + +	−	+	+ + + +	+ + + +
Anthranilic acid	±	−	−	−	−	−
Anthranilic acid conjugates	±	−	−	−	−	−

+ indicates present
− indicates absent
± indicates sometimes present

tryptophan and kynurenine. Formylkynurenine is not excreted by animals with a simple thiamine deficiency, so it becomes clear that thiamine participates in the conversion of tryptophan to formylkynurenine, i.e. at stage A . . . A in fig. 2 (Dalgliesh, 1954, 1955c). Similar experiments combining biotin and pyridoxine deficiencies show that biotin probably has no connexion with tryptophan metabolism up to the hydroxykynurenine stage (Dalgliesh, 1955b, 1955c).

4. Vitamin Deficiencies as a Research Tool

Investigations of the type described above do not necessarily prove that a particular B vitamin acts directly as part of a co-factor in an enzymic reaction. An effect may be exerted indirectly, say, by inhibiting formation of the true co-factor, or by causing an unspecific change, such as a modification in reabsorption by the kidney tubule. The technique does not therefore replace enzymic investigations, but is complementary to them. Whereas enzymic investigations show what might possibly happen, assuming the right substrate is present in the right place at the right time, study of metabolite excretion by the intact animal shows what has in fact happened. The method under discussion may not always be so readily applicable as in the above examples. It is desirable to have a rapid and specific method for the identification of relevant metabolites. Fortunately, in the study of tryptophan metabolism, this problem proved fairly easy to solve and a simple adsorption technique has been devised (Dalgliesh, 1955d), which should prove valuable in the study of human metabolism of a wide range of aromatic compounds, both endogenous (e.g. aromatic amino acid and nucleic acid degradation products) and exogenous (e.g. drugs containing aromatic rings). The in-vivo technique for investigating metabolic pathways is also likely to differ in sensitivity with different enzymes. In general there is an equilibrium

$$\text{enzyme} \rightleftharpoons \text{apoenzyme} + \text{coenzyme.}$$

A given coenzyme may be a co-factor for several apoenzymes, and affinities of these apoenzymes for the coenzyme can vary considerably. As a result, in a progressively increasing deficiency of a coenzyme the various reactions dependent on it tend to be inhibited successively, rather than simultaneously. Thus it seems that an inadequate supply of coenzyme is distributed amongst the appropriate apoenzymes preferentially, the reactions most essential to continued life of the organism being the last to suffer from the inadequate coenzyme supply. Reactions inhibited in the early stages of a vitamin deficiency are likely to be more easy to investigate.

Study of tryptophan metabolism in deficiency states has thrown valuable light on the interaction of four B vitamins. Many other cases of vitamin interactions are known. Possibly, further investigations of the type described here would show that at least some of these are due to participation of one vitamin in the formation of the active form of another. Vitamin deficiency states in man are rarely due to a simple deficiency of one vitamin. In interpreting results of replacement therapy with a single vitamin, such interactions should be taken into account.

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VITAMIN B_{12}

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1 Chemistry
2 The vitamin B_{12} group
3 Radioactive vitamin B_{12}
4 Biochemical functions
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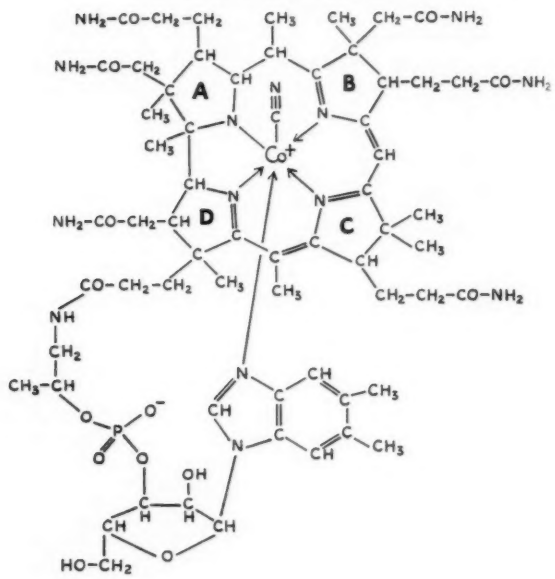
Each newly recognized vitamin has presented a difficult problem to the chemists engaged in elucidating its structure. Almost without exception, some unusual or even unique chemical feature has eventually been revealed. The story of vitamin B_{12} has certainly followed this pattern; in several respects, indeed, it is the most remarkable of them all. But perhaps it is not really surprising that biochemical catalysts should need rather special chemical structures for performing their unique vital functions.

1. Chemistry

The complete structural formula of vitamin B_{12} (cyanacobalamin, $C_{63}H_{90}O_{14}N_{14}PCo$) was first announced in July 1955 by Sir Alexander Todd and Dr Dorothy Hodgkin at a Chemical Society Symposium at Exeter. This was shortly followed by publications in *Nature* (Hodgkin, Pickworth, Robertson, Trueblood, Prosen & White, 1955; Bonnett, Cannon, Johnson, Sutherland, Todd & Lester Smith, 1955). To recount in chronological order how the complexities of this structure were unravelled would require too much space. It is simpler to work backwards, to present the formula (fig. 1) first, and then examine its separate features in turn, with particular reference to recent developments.

The molecule is built round an atom of cobalt—in itself a surprising state of affairs. No other vitamin contains a metallic atom, though metals are present in some enzymes, and in haemoglobin and chlorophyll, for example; cobalt, however, has no known place in living organisms other than as a constituent of vitamin B_{12}. The tervalent cobalt is present in a co-ordination complex that to some degree resembles a porphyrin. There are the usual four rings, each composed of four atoms of carbon and one of nitrogen, the latter being linked to the metal. However, in the haem pigments and other porphyrins the rings are joined by four single carbon atoms (methene bridges) to form a macro-ring. In vitamin B_{12} only three of these bridges are present; the fourth is missing and the rings are linked directly with no bridge at this point (between rings A and D at the left of the diagram). Also, the five-membered rings are not true pyrrols, as in the porphyrins, but are partially reduced pyrrols. The precise number (five or six) and positions of the double bonds is the only feature

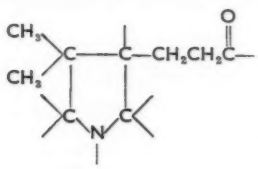
FIG. 1. TENTATIVE COMPLETE STRUCTURE FOR VITAMIN B_{12}



of the structure that remains uncertain. This unprecedented arrangement might well have eluded the organic chemists for years; it has been brought to light by *x*-ray crystallography. This remarkable achievement must be credited mainly to Dr Dorothy Hodgkin and her team at Oxford (Brink, Hodgkin, Lindsey, Pickworth, Robertson & White, 1954). The technique always involves a great many laborious calculations, but so complex is the vitamin B_{12} molecule that it has required seven years of patient and brilliant work, despite the use of mechanical and electronic calculating machines.

Side-chains are attached to all eight β-positions on the ring. As in typical porphyrins, each ring has one acetic acid and one propionic acid chain, except in ring C where a gem-dimethyl group replaces the acetic acid. Confirmation of the structure of this ring has come from the Merck laboratories in the USA, where two related degradation products have been isolated from the oxidation products of the vitamin (Kuehl, Shunk & Folkers, 1955). Both of these are believed to arise from the partial structure shown in fig. 2.

FIG. 2. FRAGMENT OF B_{12} MOLECULE



Earlier work by Karrer and his colleagues in Switzerland resulted in the isolation of a series of organic acids after vigorous oxidation of the vitamin (Schmid, Ebnöther &

Karrer, 1953). Seven acids were characterized, including dimethyl malonic acid, which could arise by further degradation of the fragments isolated in the USA. None of them contained nitrogen, however, so that no structural formula could be deduced from this work alone. The physical method, *x*-ray crystallography, is indeed uniquely valuable in revealing most clearly the central core of the molecule, which is the least accessible to chemical attack.

On the other hand, a combination of mild hydrolytic degradation with physical methods for fractionating the complex mixture of products has yielded valuable information about the periphery of the molecule. This has emerged from collaborative research by Sir Alexander Todd's team at Cambridge and by my own team at Glaxo Laboratories (Armitage, Cannon, Johnson, Parker, Lester Smith, Stafford & Todd, 1953). Electrophoresis and chromatography on paper were used to separate the series of acidic products resulting from gentle treatment with mineral acids. Interpretation of the results, including the fact that the hydrolysis is strongly catalysed by nitrous acid, suggests the presence of six carboxylic amide groups. Three of them are easily hydrolysed to the corresponding acids, and all three of these acids can be reconverted chemically into their amides, i.e., into vitamin B_{12} indistinguishable from the natural product. The removal of only one molecule of ammonia, to give a monobasic acid, is enough to destroy the vitamin activity for micro-organisms and higher animals.

The presence of aminopropanol was revealed by early investigations, and its formulation as a bridge linking the two major portions of the molecule is now confirmed. The amino group is coupled to a carboxyl on the " porphyrin-like " part of the molecule; the hydroxyl group is esterified by the phosphoric acid of the " nucleotide " portion. This part of the structure has also been known for some years: it is not that of a true nucleotide, because the base is neither purine nor pyrimidine but dimethylbenziminazole, and because the linkage to the ribose has the unusual α-configuration. The third acidic hydroxyl on the phosphoric acid was previously thought also to be esterified, but this view is no longer tenable. Instead, it appears to be free but neutralized by internal salt formation with the whole cobalt co-ordination complex, which is now seen to be basic (Lester Smith, 1955). The acidic " nucleotide " can easily be split from this basic, red, cobalt-containing portion of the molecule by brief treatment with warm concentrated hydrochloric or perchloric acid. The red portion so released turns out to be identical with " Factor B " previously isolated from calf manure; it still retains activity for a mutant of *Bacterium coli* used for microbiological assay of vitamin B_{12}, but is inactive for other micro-organisms requiring vitamin B_{12} and for higher animals, including man (Armitage *et al.* 1953).

2. The Vitamin B_{12} Group

A considerable number of closely related B_{12} vitamins have been isolated from rumen contents and faeces, sewage sludge and fermentation liquors (Ford & Porter, 1953; Friedrich & Bernhauer, 1953). Most of them are found to yield Factor B on hydrolysis with concentrated acid, so that they differ only in the " nucleotide " part of the molecule. Some of them appear to contain substituted benziminazoles but have not been fully characterized. Others contain purines instead, namely, adenine or hypoxanthine, or their 2-methyl derivatives, which have not previously been found in nature (Brown, Cain, Gant, Parker & Lester Smith, 1955). These B_{12} vitamins can also be prepared biosynthetically, by adding to a suitable fermenting system the appropriate purine or benziminazole (or a precursor thereof). The micro-organism converts this into the proper nucleotide and couples it with Factor B, which must sometimes be added in advance, to build up the B_{12} analogue. In this manner can be prepared not only the naturally occurring factors, but some new artificial ones as well. For example, a B_{12} vitamin lacking the two methyl groups on the benzene ring has been obtained, and also one in which they are replaced by chlorine atoms. There were reasons for expecting that the latter compound might be a vitamin B_{12} antagonist, but it is not; in microbiological tests it is not even inactive, but simulates vitamin B_{12} (Ford, Holdsworth & Kon, 1955; Fantes & O'Callaghan, 1955).

The biosynthesis of the natural B_{12} vitamins and their bearing on animal nutrition is discussed by M. E. Coates, S. K. Kon & J. W. G. Porter in this issue of the Bulletin (p. 61). Some of the natural and the biosynthetic factors have been tested on patients suffering from pernicious anaemia during a relapse. Factor B and the purine-containing factors, pseudo-vitamin B_{12} and Factor A, are almost if not quite inactive. Factor III from sewage sludge, containing an uncharacterized benziminazole, shows some clinical activity (Friedrich & Bernhauer, 1953; Bernhauer & Friedrich, 1954). The most active factor to date is " des-dimethyl vitamin B_{12} " lacking the two methyl groups from the benzene ring: it appears to have haematopoietic activity of the same order as vitamin B_{12} itself.

3. Radioactive Vitamin B_{12}

The preparation of vitamin B_{12} labelled with radioactive ^{60}Co, and its use in medical research, were described in a previous number of this Bulletin (Lester Smith, 1952). There are important recent developments in this field. ^{60}Co is not the most suitable cobalt isotope for the purpose: although readily available at low specific activities, it is almost impossible to obtain the high specific activities needed for some investigations. Also the relatively long half-life of 5.3 years severely limits the total safe dose for any subject. Accordingly, other isotopes have been tried. ^{58}Co (half-life 72 days) can be made by transmutation of nickel in the atomic pile; in theory this isotope can be made "carrier-free", but in practice the specific activity was depressed by traces of cobalt in the nickel target material (Bradley, Lester Smith, Baker & Mollin, 1954). More recently, in collaboration with Doctors D. L. Mollin and J. E. Bradley at the Postgraduate Medical School of London, cyclotron bombardment of iron has been used to prepare mixtures of ^{56}Co (half-life 72 days) and ^{57}Co (half-life 270 days) in carrier-free condition. This material has yielded labelled vitamin B_{12} with the phenomenally high specific activities of 12 c/g. and 13.5 c/g. New fields of research are thereby opened up; for example, a total dose of about 5 μc may safely be given, so that a series of tests can be made on a single subject, who thus serves as his own control. Again, an oral dose of a fraction of a microgram leads (in a normal subject, but not in a case of pernicious anaemia) to a serum level of vitamin B_{12} readily measurable directly by a

scintillation counter (Mollin & Baker, 1955; Mollin & Lester Smith, 1956). This promises to provide a technique for diagnostic purposes and for assay of intrinsic factor preparations simpler and more direct than the faecal or urinary excretion tests or the hepatic uptake tests. The paper by L. J. Witts in this issue of the Bulletin (p. 14) describes these techniques and the knowledge of the absorption of vitamin B_{12} gained by their use.

4. Biochemical Functions

Vitamin B_{12} seems to be involved in the metabolism of proteins, fats, carbohydrates and nucleic acids. Yet this bewildering complexity of function presumably ought to stem from the promotion of a single enzymic reaction, or at most of a few related reactions. This argument derives from the extremely small requirements of B_{12}, which surely point inexorably to its catalytic function as the prosthetic group of one or more enzymes. Just as coenzymes I and II each link loosely with several different proteins (apoenzymes) to form a series of dehydrogenase enzymes, so might vitamin B_{12} function in more than one system. However, no enzyme containing vitamin B_{12} has yet been identified, and these ideas are still a long way from being firmly established. Nevertheless it does seem useful to see whether present knowledge can be tentatively integrated within this framework, bearing in mind that some of the speculations may be disproved by future research. What already does seem likely, although the idea has not been clearly enunciated, is that vitamin B_{12} brings about the reduction of some biochemically important molecules. This is no surprising function: the molecule contains tervalent cobalt, which can be reduced fairly readily to the bivalent state, and the product is then a strong reducing agent; it will in effect reduce oxygen, becoming itself reoxidized to the original vitamin B_{12} by contact with air. The vitamin exists normally in the higher state of oxidation, so that one might rather expect it to show oxidizing powers. However, there is no real distinction when it is functioning catalytically in a biochemical system, for, if one molecule is to be reduced, it must be at the expense of another that is oxidized. Moreover the metal is co-ordinated in such a manner that some of the attached groups (including the cyano group) can readily be replaced by others without disrupting the molecule: such changes are probably involved in its linkage to the apoenzyme and could easily modify the tendency to become reduced, i.e., the redox potential, to bring it within the physiological range.

For simplicity of presentation, the recent evidence that vitamin B_{12} operates in this fashion will be briefly reviewed first; it can then be seen how the earlier established nutritional functions and the pathology of vitamin B_{12} deficiency could follow logically from this scheme.

A number of investigators, using either growth response or isotopic tracer techniques, have clearly demonstrated that vitamin B_{12} mediates the *de-novo* synthesis of labile methyl groups; this is true for several animal species and some micro-organisms. The precursors of the methyl carbon atom are formate, the β-carbon of serine or the α-carbon of glycine; the new methyl groups first appear in methionine. They may then be transferred to become the methyl groups of choline or creatine, but, contrary to earlier indications, vitamin B_{12} is not involved in this transmethylation reaction (reviewed by Arnstein, 1955; Lester Smith, 1954). The acceptor molecule is known to be homocysteine, which may first need to be reduced from its disulphide form, homocystine. There is indeed evidence (see below) that vitamin B_{12} may bring about the reduction of this and other —S—S— compounds. The next steps are presumed to be the transfer of "formyl" or "hydroxymethyl" to the sulphur atom, followed by the reduction of either product to the $—S—CH_3$ compound, namely, methionine. Thus two reductive steps are involved and it seems probable that the vitamin B_{12} enzyme system brings about one or the other, if not both.

A closely analogous reaction is the methylation of the pyrimidine, uracil, to yield thymine. A precursor is again the hydroxymethyl group of serine, so that the intermediate may well be hydroxymethyluracil. Vitamin B_{12} could then be involved also in the reduction of this hydroxymethyl group to methyl, thus affording one possible explanation of the part it is known to play in nucleic acid synthesis. The isolation of hydroxymethyl cytosine from a bacteriophage affords some justification for the postulated intermediate.

It is true that folic acid is also concerned in the conversion of uracil to thymine or thymidine, but the two hypotheses are not mutually exclusive. There are good reasons for believing that folic acid takes part in the transfer of one-carbon units; if so, it may well bring about the hydroxymethylation step, and vitamin B_{12} the reduction step or the coupling with deoxyribose to form the thymidine component of deoxyribonucleic acid (DNA). One must also recall that much of this investigation into metabolic pathways has been done on micro-organisms, and not on higher animals; alternative routes, one by way of folic acid, the other by way of vitamin B_{12}, may well be available in different species or even within the same organism.

Whatever the precise mechanism, the methylation of uracil is evidently of prime importance in the bone-marrow abnormalities of pernicious anaemia. In relapse the ratio of uracil to thymine in the marrow is raised greatly above normal, as is the corresponding ratio of ribonucleic acid (RNA) to DNA. On treatment, however, whether with vitamin B_{12} or with folic acid, these ratios fall rapidly to normal: moreover, this precedes the reticulocyte response. A suggested interpretation is that the treatment catalyses the synthesis of the thymine that appears in the DNA of the new red cells (Glazer, Mueller, Jarrold, Sakurai, Will & Vilter, 1954). Earlier work (Davidson, Leslie & White, 1948; White, Leslie & Davidson, 1953) showed, however, that the amounts per gram or per cell of DNA and especially of RNA were abnormally high in megaloblastic marrows. On treatment the RNA content fell the more quickly, which would lead to the changed ratios observed by Glazer *et al.* (1954). Since DNA occurs in cell nuclei and is involved in their division, disturbance of its synthesis is felt first in cells with frequent mitoses, namely, those of the bone-marrow, gastrointestinal tract and epithelium. This then leads to the megaloblastic anaemia, leucopenia and glossitis of pernicious anaemia. Contrary to previous ideas, the typical gastric atrophy may possibly also arise as the effect and not the cause of impaired absorption of vitamin B_{12}: the primary cause would still be the failure to secrete adequate amounts of intrinsic factor.

To support the suggested function of vitamin B_{12} in maintaining thiol compounds in the reduced state are these observations: in deficient rats, and also in patients with pernicious anaemia, the blood levels of thiols (mainly as glutathione) are subnormal and rise to normal on administration of vitamin

B_{12}; no such effects are noted from deficiencies of folic acid or iron (Ling & Chow, 1953; Register, 1954). Similarly the blood level of another reducing substance, ascorbic acid, is often low in pernicious anaemia; moreover, in five of seven pernicious anaemia patients, injected ascorbic acid was promptly oxidized to dehydroascorbic acid (measured in the plasma). After treatment they behaved like normal subjects and showed raised plasma levels of ascorbic acid itself after its injection (Will, Mueller, Glazer, Friedman & Vilter, 1953).

It has been postulated that glutathione in the reduced state serves to reactivate —SH enzymes that may have become oxidized to the inactive —S—S— forms during vitamin B_{12} deficiency (*Chemical and Engineering News*, 1953). Thus, in chronic vitamin B_{12} deprivation of rats or chicks, fat accumulates in the tissues because, it is suggested, oxidation of coenzyme A to its ineffective disulphide form inhibits the normal metabolic pathway (see also Boxer, Ott & Shonk, 1953). This is independent of the lipotropic action of vitamin B_{12} exercised through synthesis of the methyl group of choline. Several —SH enzymes participate also in carbohydrate metabolism, and their reactivation by vitamin B_{12} could explain in part its growth-promoting effect. More specifically it may function by this route in promoting the synthesis of ribose and deoxyribose or their incorporation into nucleic acids. It has been claimed, for instance, that vitamin B_{12} stimulates the conversion of glucose to ribose in erythrocytes (Ling & Chow, 1954). This is therefore a second way in which the vitamin may be involved in biosynthesis of the nucleic acids; moreover, both types of nucleic acid may arise by this route. In support of this possibility, the livers of rats deficient in B_{12} were found to contain subnormal amounts of both RNA and DNA (Schweigert, Scheid & Downing, 1954).

To return to protein metabolism: there are again two ways in which it may be influenced by vitamin B_{12}. Methionine synthesis is undoubtedly inhibited by lack of the vitamin, so that on a methionine-poor diet there would be a shortage of this essential amino acid. This would diminish the synthesis of body proteins, and other amino acids would be excreted (Weaver & Neill, 1954) or wastefully catabolized. Moreover, whether by this mechanism or by some other involving inhibition of an —SH system, one can envisage abnormal metabolism of tryptophan and tyrosine; this in turn could lead to the excessive urinary excretion of phenolic bodies noted in pernicious anaemia, and also perhaps to the formation of toxic substances causing the haemolysis that sometimes accompanies the disease. Both metabolic abnormalities, when present, disappear dramatically after injection of vitamin B_{12}. The second postulated mechanism operates by inhibiting the synthesis of RNA, which in turn is supposed to act as a "template" for the building of specific protein molecules (Hsu, Stern & McGinnis, 1953). The effects of this inhibition would be felt first in cells with a high RNA content and rapid protein turnover. Niewig, Faber, de Vries & Kroese (1954) have pointed out that just such conditions hold in the axon of the nerve cell. The adult nerve cell does not divide, DNA turnover is low, but RNA content is high, and the integrity of the axon is maintained only by intense protein synthesis. Degeneration sets in if this is prevented and, accordingly, the nerve cells most affected in subacute combined degeneration of the spinal cord are precisely those with the longest axons.

To explain on this basis why folic acid cannot correct the neurological manifestations of pernicious anaemia, we must assume that vitamin B_{12} is essential and irreplaceable for RNA synthesis. To explain why folic acid without vitamin B_{12} can at high levels control, at least for a time, the haematological symptoms, one must make one of two further assumptions. Either folic acid can by itself bring about DNA synthesis, perhaps using up preformed intermediates in the process, or else both factors are needed for its synthesis, but large doses of folic acid serve to mobilize small residual reserves of vitamin B_{12} from storage in the liver or other depots; when these are finally consumed, folic acid becomes ineffective, in accordance with the experience of some haematologists. Or, more immediately, a level of vitamin B_{12} too low to be ordinarily effective may be reactivated for DNA synthesis by the mass action of an abnormally high level of folic acid through the interlocking steps of enzymic synthesis controlled by the two factors. Thus, suppose some precursor A is converted by folic acid into an intermediate B, which is carried forward to DNA by vitamin B_{12}, as in the reactions:

$$A \xrightarrow{FA} \mathbf{B} \xrightarrow{B_{12}} DNA$$

A high level of folic acid (FA) speeds up the first reaction leading to an abnormally high concentration of B (indicated in the diagram by heavy type). The second reaction is slowed down by inadequate vitamin B_{12} (indicated by light type), but enough DNA is formed because there is plenty of the intermediate. Similar reasoning can be applied if the second reaction is catalysed by folic acid and the first by vitamin B_{12}, especially if this step is reversible, as indicated in the following reactions:

$$A \overset{B_{12}}{\rightleftharpoons} B \xrightarrow{FA} DNA$$

No such mechanism will stimulate RNA production in a state of vitamin B_{12} deficiency if its synthesis is mediated by this vitamin alone.

This hypothesis seems reasonable, because in deficiency states the shortage of the vitamin is relative, not absolute; in severely relapsed cases of pernicious anaemia, for example, the serum B_{12} level is still around one-tenth of the normal figure (Mollin & Ross, 1953). Thus enzyme reactions will be slowed down, not halted; complete stoppage probably means death.

There is evidence both for and against the idea that vitamin B_{12} converts stored folic acid into its functionally effective form, folinic acid; if true, it is another example of a reducing function of the vitamin, for folinic acid is reduced formyl folic acid (Doctor, Welch, Perrett, Brown, Gabay & Couch, 1953).

Thus, from the plausible assumption that vitamin B_{12} functions as the prosthetic group of at least one reducing enzyme, the rest can be argued logically: namely, its involvement in the metabolism of fat, protein and carbohydrate and, along with folic acid, of both types of nucleic acid. Consequently metabolism of all major nutritional elements may become deranged in vitamin B_{12} deficiency. Its appetite-stimulating and growth-promoting effects in conditions of mild deficiency are therefore not hard to explain, besides its more dramatic effects in several forms of megaloblastic anaemia.

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VITAMIN C

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1 Specificity
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2 Methods of estimation
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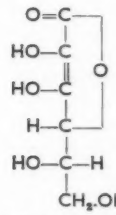
The object of this article will be to attempt to summarize, in brief outline, the present status of our knowledge of the antiscurvy vitamin. It will not be our purpose to concentrate our attention too exclusively upon the papers published within the limits of the last two or three years only. A treatment of that kind is in fact already available in numerous useful review articles and annual reports (e.g. *Nutrition Abstracts & Reviews*; *Nutrition Reviews*; *Annual Reviews of Biochemistry*). Here our aim will be rather to assess the implications of the newest findings in the light of the accepted knowledge that has gradually accumulated during the past two or three decades. Particularly, an effort will be made to draw attention to the numerous gaps in our knowledge as well as to the established facts.

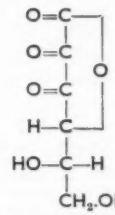
1. Specificity

a. One Natural Form Only

Vitamin C is unique (or almost unique) among the vitamins in the fact that only one form, L-ascorbic acid, is known to occur in nature. It is true that, as such, it can exist in two states of oxidation, namely (i) as the reduced form, L-ascorbic acid itself, accounting for the great bulk of the vitamin as found naturally in animal and vegetable tissues, and (ii) as the reversibly oxidized form, dehydroascorbic acid, of which small amounts only can be found in nature. Nevertheless, this great contrast remains to distinguish vitamin C sharply from the other vitamins, most of which exist in a considerable variety of different active forms. (To take one example, vitamin A occurs as vitamin A_1, vitamin A_2, and various active carotenoid pigments. Similar considerations apply to vitamins D, E, K, and so on.)



L-Ascorbic acid



Dehydroascorbic acid

b. Coenzyme Complexes and " Combined Forms " Not Known

Another distinction is that most of the other water-soluble vitamins are now known to function biologically in the form of various complex nucleotide systems, or coenzymes, as components of a series of different enzymes. Thus, we have the two pyridine coenzymes, containing nicotinamide; the numerous flavoprotein enzymes, containing riboflavin; the vitamin B_6 systems; the pantothenic residue in the coenzyme A complexes; etc. But ascorbic acid has not so far been definitely shown to be involved in any coenzyme complex of this nature. Nor, for that matter, has any " combined form " of vitamin C ever been isolated, although theories as to their existence have not been lacking.

c. Artificial Analogues

Although a considerable number of substances closely related to vitamin C have been prepared artificially, and although several of them possess some degree of biological activity[1], it is notable that no such compound has ever been demonstrated to occur in nature.

2. Methods of Estimation

a. Biological

Biological methods of assay for vitamin C have in recent years been largely replaced by chemical methods. Indeed biological tests are used nowadays only when there is some special need for checking the chemical test. The standard biological technique employed in the early days of research on vitamin C depended on the prevention of scurvy in guinea-pigs (Holst & Frölich, 1912; Sherman, LaMer & Campbell, 1922). This was largely replaced by a simpler growth test (see Harris & Olliver, 1942). A second standard biological method, which has the advantage of being highly specific for vitamin C, is based on a microscopic examination of tooth structure (Höjer, 1926; Key & Elphick, 1931). Several other procedures have been proposed for biological assays (e.g. one based on a measurement of serum " alkaline " phosphatase), but have not come into general use.

b. Chemical

The technique still most commonly used for chemical determination of vitamin C consists of a decolorization of indophenol dye, carried out in acid reaction within a specified

[1] The work in this field has been summarized by F. Smith (1954).

and brief time-limit (Harris & Ray, 1933a; Birch, Harris & Ray, 1933; Harris, 1937; Bessey & King, 1933). Trouble from interfering substances is not commonly encountered, and can be allowed for by various means when they are present (see, for example, Harris, Mapson, Kodicek, Moore & Booth, 1947; Olliver, 1954). The second most commonly used technique involves the use of an osazone reaction (Roe & Kuether, 1943). Various other reagents have been proposed in recent years, but none has been widely accepted.

c. Microbiological

It is a remarkable fact that vitamin C is almost the only water-soluble vitamin for which no generally accepted microbiological method of assay has yet been devised. None the less, it is known that certain bacteria can intervene in vitamin C metabolism, in, for example, the ascorbic-dehydroascorbic equilibrium.

3. Distribution

The outstanding puzzle about the distribution of vitamin C is its apparent haphazard occurrence in the plant and animal kingdoms. Some fruits and vegetables are richly endowed with vitamin C (e.g. black currants, water-cress, Brussels sprouts, guava, horse-radish, green peppers); others, apparently not essentially dissimilar, contain relatively little (e.g. pears, celery, mushrooms, pomegranates, plums, beetroot, damsons, figs). No explanation can yet be offered. Again, it remains a mystery, teleologically speaking, why vitamin C is present in high concentrations in some animal organs (e.g. the adrenals, eye lens, vitreous and aqueous humour, white blood cells) and not in others (red cells, muscle, brain, pancreas).

4. Scurvy in Experimental Animals

The only species yet shown to need vitamin C are primates (men and monkeys) and guinea-pigs. Many farm and domestic animals, birds, and wild beasts are known to synthesize the vitamin in the body. Little information is yet available to account for the species difference.

Perhaps the two most characteristic features of experimental scurvy in guinea-pigs are: (i) the occurrence of haemorrhages, and (ii) the loss of activity of the functional cells, this manifesting itself in the failure of collagen formation and in the cessation of normal formation of new bone and tooth structures.

It is noteworthy that no adequate biochemical explanation of these structural changes can yet be offered (see section 9b).

One result of vitamin C deficiency in guinea-pigs, to be referred to again later, is the enlargement of the adrenals (Rondoni, 1919; Höjer, 1924).

5. Scurvy in Human Beings

i. *Occurrence.* Although it is often stated that scurvy is now to be seen only in pathological museums, it is clear from several recently published papers that crops of cases may still be encountered from time to time, both among children and adults, and in the USA (Follis, Park & Jackson, 1950), as well as in Britain (Howells, Palmer & St John-Brooks, 1954) and elsewhere (Grusin & Kincaid-Smith, 1954).

ii. *Characteristics.* Recent work has done little to add to the classical description of the signs and symptoms, or the morbid anatomy, of scurvy, notably the haemorrhagic changes in adults and the bony abnormalities in infants. Some doubt must remain whether the so-called anaemia of scurvy is a real and distinct entity (cf. section 9a (ii)).

In the course of the very carefully controlled observations on the production of vitamin C deficiency in human volunteers at Sheffield (Medical Research Council, 1948), a specially remarkable feature was the occurrence of cardiac emergencies in at least two subjects. Another subject suffered from a recurrence of a latent tuberculous spondylitis. The old observation, that, in scurvy, scars become red and livid, was confirmed. The earliest lesion that was noted in these studies at Sheffield was the occurrence of a follicular hyperkeratosis; and an entirely new finding was a very pronounced exacerbation of any tendency to acne.

6. The Human Requirement

There has been some confusion in the past about the apparent discrepancy between the various standards. The League of Nations (1938) "standard of requirement" was 30 mg./day for an adult, and the "desirable intake" as recommended by various American authorities, e.g. National Research Council (1948), varies from 50 mg. to 75 mg./day.

In the Sheffield trials, it was found that "the 'minimum protective dose' of vitamin C, as measured by the criteria of the presence of scurvy, was in the region of, perhaps somewhat below, 10 mg. daily" (Krebs, 1953).

It has to be borne in mind, however, that guinea-pigs are known to need considerably more vitamin C for normal physiological function (e.g. adequate odontoblast production) than for mere protection against overt scurvy: what we do not know is the relation in man between the optimum requirement and the minimum (scurvy-preventing) requirement (see Harris, 1953b).

7. Assessment of Status

The method introduced by the writer and his colleagues for assessing the nutritional status of human subjects in vitamin C consists of giving daily test doses ("loading" doses) of vitamin C by mouth, and counting the number of days until a surplus begins to overflow in the urine. (See Harris (1942, 1943), and earlier literature there cited.) The lower the past intake of vitamin C has been, the poorer the degree of saturation of the tissues, and hence the greater the amount of vitamin C so needed. This was the first of the methods of assessing vitamin status; and corresponding saturation tests have since been introduced for various other vitamins as well.

Vitamin C status can be measured also by a determination of ascorbic acid in the blood (preferably in the white-cell layer), and the results seem to run in line in a general way with those obtained by the saturation test.

8. Biosynthesis

Until recently little was known about the precursors of vitamin C in plants or animals, or the site of its formation. We now have evidence (Isherwood, Chen & Mapson, 1953; Mapson & Isherwood, 1955; Horowitz & King, 1953) that the pathway is as follows:

D-glucose ⟶ D-glucurono-γ-lactone ⟶ L-guluno-γ-lactone ⟶ L-ascorbic acid

By an analogous sequence, D-galactose can also function as a precursor:

D-galactose ⟶ D-galacturonic acid ⟶ L-galactono-γ-lactone ⟶ L-ascorbic acid

The most remarkable feature is the change from the D- to the L-series at the second step.

It is thought that animals such as man and guinea-pigs, that need exogenous vitamin C, lack the enzyme (or enzymes) needed to bring about this second step.

9. Mode of Action

A detailed appraisal of the known facts relating to the problem of the mode of action of vitamin C has recently appeared elsewhere (Harris, 1953a). Here we can do no more than allude very briefly to some of the most salient points.

a. Biochemical Role

i. *Tyrosine metabolism.* It is well established that ascorbic acid, in vivo and in vitro, promotes the oxidation of L-tyrosine (or of phenylalanine). (For references to literature, here and below, see Harris, 1953a.) This is perhaps the most suggestive of the known chemical effects of the vitamin; unfortunately, however, this reaction in itself is inadequate to account for the high specificity of L-ascorbic acid as preventer of scurvy, since various other substances, some of them quite without antiscorbutic activity, can also stimulate tyrosine oxidation in an analogous way (see, for example, Guggenheim, Leibowitz & Rakover, 1953; *Nutrition Reviews*, 1954).

ii. *Folic acid metabolism.* Ascorbic acid is concerned, likewise, in a biochemical system that converts folic acid (pteroylglutamic acid) to a closely related substance, folinic acid (or to the so-called "citrovorum factor"). But, once again, it cannot be shown that this fact has any specific bearing on the role of ascorbic acid as the unique antiscurvy vitamin. It may, however, be expected to lead us eventually to a better understanding of a possible connexion, suspected for many years, between scurvy and anaemia.

iii. *Reducing action, etc.* Because of its intense reducing properties, and in particular the presence in the molecule of the remarkable enediol grouping, it may reasonably be supposed that the secret of vitamin C action lies in oxidation-reduction reactions in the body. It is true that the action of vitamin C has been investigated in many diverse directions, both in the intact animal and in isolated systems (for review see Harris, 1953a), in relation, for example, to glutathione, haemoglobin, tissue respiration, enzyme activity, and so on: nevertheless it must be admitted once again that a general lack of specificity is evident in all these connexions.

iv. *Plant biochemistry.* Studies have been made of the influence of vitamin C on such reactions as the following: lactate-pyruvate oxidation (James & Cragg, 1943), the triose-phosphate system (James, Heard & James, 1944), tyramine-quinone equilibrium (Miller, Mallette, Roth & Dawson, 1944). It remains true nevertheless that the essential function of vitamin C in plant physiology is virtually as obscure as ever. (For review see Mapson, 1953.)

b. Morphological Control

According to my view, the structural abnormalities seen with deficiency of vitamin C, notably in the growing teeth and the bones, can be attributed to an impairment in the functional activity of the formative cells (Harris, 1953a). On this basis can be explained the failure of production of dental enamel and cementum, and the fact that the dental abnormalities seen with a prolonged partial deficiency of vitamin C are in some respects more remarkable than those with a complete deficiency (Fish & Harris, 1934). Likewise the cessation of osteogenesis, the defective formation of collagen (Wolbach & Howe, 1926), and the delayed healing of wounds can be interpreted along the same lines.

The reason for the vascular breakdown, resulting in the most typical sign of scurvy, the occurrence of haemorrhages, has not yet been adequately accounted for.

10. Vitamin C and Adrenal Function

An immense and somewhat controversial literature has grown up on the question whether some intimate relationship exists between vitamin C and adrenal function. The facts suggesting some such connexion are briefly as follows.

a. Apparent Interrelations

i. Vitamin C, at that time known as hexuronic acid, was in fact originally isolated from the adrenals, as well as from plant sources (Szent-Györgyi, 1928). It is present in high concentrations, both in the cortex of the adrenal (Harris & Ray, 1932, 1933a) and—in an amount not much less—in the medulla (Harris & Ray, 1933b).

ii. Vitamin C deficiency results in an enlargement of, and in characteristic pathological changes in, the adrenal glands (section 10b).

iii. An injection of adrenocorticotrophic hormone (ACTH) is followed by a transient fall in the concentration of ascorbic acid in the adrenals (Sayers, Sayers, Lewis & Long, 1944).

iv. Claims have also been made that the vitamin C requirement can be influenced by adrenal hormone or ACTH, or that these are further affected by exposure to stress or to cold, and so on. (For reviews, see Pirani, 1952; *Nutrition Reviews*, 1950; Bessey, Lowe & Salomon, 1953.)

b. Limited Significance

As against the assumption of a fundamental and all-important interrelation, we may recall that Höjer, as long ago as 1924, remarked that the changes in the adrenals of scorbutic guinea-pigs did not appear earlier than those in other parenchymatous tissues, and added: "These changes must, therefore, by no means be taken as a reason for placing the adrenals in the centre of the pathology of scurvy". In the same direction, Reid (1954) in her review of the published work on the relation of vitamin C to the adrenals cogently concludes:

> Although much study has been made of the relation of vitamin C to the adrenals, the time has not arrived for drawing definite conclusions as to its role in the production of corticosteroids. However, the burden of proof appears to rest on those who hold that it has a positive effect.

c. Newer Findings

My colleagues and I, in a series of somewhat elaborate experiments, came to the following conclusions (see Harris, Bland, Hughes & Constable, 1953; Cherry, Bland, Constable, Hughes & Harris, 1954; Harris, Constable, Hughes & Loewi, 1955; Constable, Harris & Hughes, 1955): while the continued administration of cortisone or of ACTH to guinea-pigs did cause an appreciable, localized, diminution in the

concentration of ascorbic acid in their adrenals, it was nevertheless without measurable effect on the over-all vitamin C requirements of the animals, as measured either by dental histology or by survival periods. Again, the enlargement of the adrenal caused by ACTH, or its diminution caused by cortisone, was not governed by the animal's vitamin C intake. The continued administration of cortisone or ACTH resulted (in guinea-pigs and rabbits, but not in some other species) in a massive enlargement of the liver which was associated with an augmented glycogen deposition, and an increased cell size: this, however, was not related to the vitamin C intake.

11. Vitamin C and Infection

Two facts stand out. First, it is universally recognized that vitamin C deficiency in guinea-pigs carries with it, almost inevitably, the liability to infection; in human beings also, scurvy is generally believed to lower the resistance to infection. Secondly, in infectious ailments, notably so in pulmonary tuberculosis (Abbasy, Harris & Ellman, 1937), there is a greatly lowered vitamin C status as indicated by the results of saturation tests; furthermore the tissues of infected animals are found on analysis to have a lowered ascorbic acid content (Harris, Passmore & Pagel, 1937), indicating presumably an increased usage, or loss, of the vitamin. One may legitimately speculate whether in the absence of vitamin C there is some impairment in the production of the disease-fighting mechanisms, just as there is a failure of the formative cells to produce new body tissues in general. There are, in fact, suggestions that phagocyte activity can be stimulated by vitamin C (Meyer & McCormick, 1928; Nungester & Ames, 1948).

12. Conclusions

From what has been said above, it will be agreed that among the more important problems awaiting further attention are the following:

i. a knowledge of the specific biochemical systems intrinsically responsible for the antiscorbutic action of vitamin C;

ii. a re-examination of several microbiological systems known to influence ascorbic acid metabolism;

iii. the puzzle of its apparently somewhat haphazard distribution in animal and plant life;

iv. a better understanding of the relations between vitamin C and the adrenals;

v. an explanation of the extreme liability to infection seen in deficient animals;

vi. evidence of the existence of hypovitaminosis, as distinct from avitaminosis, in human beings (comparable with the data that already exist for guinea-pigs); hence a knowledge of the optimum, as apart from the minimum, human requirement.

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VITAMINS IN ANIMAL NUTRITION

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It is clearly beyond the scope of this article to attempt to review the vast amount of work that has been published during the last few years on the subject of vitamins in animal nutrition. Accordingly we have made a somewhat arbitrary choice of topics, selecting those having a direct bearing on problems of practical interest to workers on animal nutrition, and deliberately ignoring those investigations in which the animal has been used primarily as a tool in the elucidation of the biochemical function of vitamins.

The importance of vitamins as accessory food factors is well known ; signs of deficiency are generally familiar and the recommended dietary requirements have been laid down for man and for many species of animals. In recent years, however, it has become increasingly evident that in practice the dietary requirement of an animal for any vitamin is not constant under all conditions. For instance, the biosynthetic activity of the intestinal micro-organisms may make an appreciable contribution to the animal's needs for certain vitamins. Conversely, various factors may prevent the vitamins known to be present in the food from being completely available to the animal. Examples of each of these circumstances are considered in this article.

1. Intestinal Synthesis of Vitamins

a. General

Intestinal synthesis of vitamins of the B complex and vitamin K has now been demonstrated in several species of animal. In ruminants synthesis occurs also in the rumen before the food enters the true digestive tract and passes through its absorptive part, so that conditions are exceptionally favourable for the efficient utilization of the products. Adult ruminants are practically independent of an exogenous supply of vitamins of the B complex. In fact, the only observed deficiency of a water-soluble vitamin is of vitamin B_{12} (see below). In non-ruminants the main site of microbial activity is the large intestine and, although much microbial synthesis is known to occur there, the products of such synthesis are poorly utilized by the host. Thus the contribution to the animal's needs is usually only relatively small. Sometimes, however, animals are known to eat their faeces, and when they do so they derive considerably more benefit from the products of microbial synthesis. The requirements for some vitamins, as, for example, vitamin B_{12} and possibly some as yet unidentified factors, may be completely satisfied by this means.

b. Vitamins of the B Complex in Ruminants

The requirement for the vitamins of the B complex in the ruminant is closely associated with the development of the digestive tract. A new-born calf may be considered as essentially an animal with a single stomach; the rumen is small but begins to grow rapidly as soon as the calf starts taking solid food, until, after two or three months when the calf is weaned, the rumen has become fully functional.

Although synthesis of vitamins in the rumen has been recognized for some 40 years, its importance to the ruminant is even now not clear since its extent varies with the ration. Comparisons of the vitamin content of the rumen with that of the diet, or measurement of the urinary and faecal excretion of vitamins, have demonstrated synthesis of all the members of the vitamin B complex (cf. Kon & Porter, 1954). Such findings are essentially qualitative, for it is extremely difficult to measure the total amount of any vitamin synthesized in a dynamic system such as the rumen: the products of synthesis are continually passing along the digestive tract, and it is also possible that vitamins may be absorbed directly from the rumen.

Interest in recent years has naturally centred on the quantitative aspects of vitamin synthesis, and Kon & Porter (1953) and Agrawala, Huffman, Luecke & Duncan (1953) have shown that the concentration of vitamins in the rumen contents varies relatively little whether the diet is rich in vitamins or almost devoid of them. These findings indicate a balance between the extent of synthesis and absorption of vitamins by the microbial flora and their concentration in the rumen.

c. Cobalt and the Biosynthesis of Vitamin-B_{12}-Active Compounds in the Gut

Deficiency of cobalt leads to a wasting disease in ruminants, recognized and described in various areas in many parts of the world (see Marston, 1935; Underwood & Filmer, 1935). The earlier work on the role of Co showed that it was effective only when given by mouth (cf. Marston, 1952); Tosic & Mitchell (1948) showed that Co was concentrated by the micro-organisms in the rumen, suggesting that this was the site of its function. When vitamin B_{12} was isolated and shown to contain Co it occurred to a number of workers to examine its effect on Co-deficient ruminants, and it has become clearly established that vitamin B_{12} by mouth or by injection cures animals with the signs of Co deficiency (cf. Kon & Porter, 1954).

During a comparison of methods of measuring vitamin B_{12} in rumen contents and faeces of steers, Coates and her co-workers (Coates, Ford, Harrison, Kon, Porter, Cuthbertson

& Pegler, 1951; Coates, Ford, Harrison, Kon & Porter, 1953) found that the apparent potency for micro-organisms was substantially greater than that for chicks. Following up this finding, Ford, Kon & Porter (1952) and Ford & Porter (1952, 1953) described the separation of the vitamin B_{12} activity of rumen contents and faeces into vitamin B_{12} and three other factors, A, B and C. Subsequently, with the application of improved techniques, especially of ionophoresis (Holdsworth, 1953), several more factors were shown to be present (cf. Kon, 1955). These compounds are related to, but are not members of, the cobalamin series. Those of known structure are derived from Factor B, and differ from vitamin B_{12} only in that various purine bases replace 4:5-dimethylbenziminazole in the nucleotide portion of the molecule (see Lester Smith in this number of the Bulletin, p. 52).

These factors show activity comparable with that of vitamin B_{12} in microbiological tests with *Bacterium coli* or *Lactobacilli*, but of the twelve so far tested here only one, vitamin B_{12III} (Friedrich & Bernhauer, 1953), shows activity for *Ochromonas malhamensis* or for higher animals (Coates & Ford, 1955). Whereas the gut contents and faeces of animals contain these factors, usually in considerable excess of their cyanocobalamin content, animal tissues contain predominantly cyanocobalamin with only traces of other factors (cf. Kon, 1955). Thus the role of these factors in animal nutrition is, at best, indirect in that they may act as growth factors for rumen or other intestinal micro-organisms.

d. Unidentified Growth Factors

Much evidence has been presented in recent years for the existence of growth factors other than vitamin B_{12} in many natural materials. Most of the work has been done with chicks, probably because their early growth is extremely rapid and vitamin inadequacies are rapidly shown up. In spite of many attempts no one has succeeded in isolating any of the postulated factors. Menge, Combs, Hsu & Shorb (1952) have reviewed the subject extensively and have presented evidence for the existence of at least two growth factors for chicks, one in liver and yeast and a second in whey. Recently Scott, Morrison & Norris (1955) have suggested that the active principle of such materials as whey, grass-juice and fish solubles persists in the ash and is probably a mineral. Much of the work in this field has been done using synthetic or semi-synthetic diets, and it is possible that on such diets the animal's requirements for some of the known nutrients may be higher than is realized. If so, supplements of natural materials may well cause a growth increase and hence appear to provide unknown growth factors. On the other hand, it is conceivable that certain unknown growth factors are normally synthesized by intestinal micro-organisms and are available to the host; on artificial diets synthetic activity may be altered and less of these factors is produced. The problem of the need for further growth factors has been approached by experiments with germ-free animals (Reyniers, Trexler, Ervin, Wagner, Gordon, Luckey, Brown, Mannering & Campbell, 1950). These workers reared chicks in germ-free conditions on synthetic diets consisting of purified ingredients and the known vitamins in adequate quantity. The birds grew normally throughout the experiments, which lasted for 4–8 weeks, and their growth was not improved by supplements of natural materials containing the supposed unidentified factors. Thus there was no evidence that the diet was deficient in unknown vitamins or that micro-organisms in the intestine contributed any such factors. However, as the authors point out, unidentified nutrients might possibly have accompanied some of the dietary constituents or, more likely, the chicks carried sufficient reserves of essential factors to last through the first few weeks of life.

e. Effect of Antibiotics and Other Antibacterial Agents on Intestinal Synthesis of Vitamins

Recently much interest has centred around the discovery that antibiotics given in the diet increase the growth of young animals, and considerable speculation as to their mode of action has been aroused. Since other antibacterial agents, such as sulphonamides, detergents and organic arsenicals, have also been reported to have growth-promoting properties, it is a reasonable assumption that the primary action of these drugs is on the bacteria either of the gut or of the environment. There are many reports, among them those of Romoser, Shorb, Combs & Pelczar (1952), Anderson, Slinger & Pepper (1953) and Quinn, Story, Catron, Jensen & Whalen (1953), that antibiotics given in the diet cause alterations in the microbial population of the gut. Unfortunately, findings in different laboratories are not consistent with each other, and it has not been possible to correlate distinct microbial types with particular nutritional effects. There is much evidence that certain vitamins, particularly of the B complex, are "spared" by antibiotics. Most of it is based on laboratory experiments with rats or chicks on diets containing suboptimal amounts of vitamins. Thiamine appears to be the vitamin most readily spared by antibiotics (Lih & Baumann, 1951; Waibel, Cravens & Baumann, 1953), but economies in folic acid, biotin or riboflavin have also been claimed (Biely & March, 1951; Coates, Dickinson, Harrison & Kon, 1951; Stokstad & Jukes, 1951). Luckey, Pleasants & Reyniers (1955) have reported that in germ-free chicks dietary deficiencies of thiamine, riboflavin, nicotinic acid and folic acid were more pronounced than in conventionally-kept chicks. It is, therefore, reasonable to suppose that chicks normally make some use of vitamins synthesized by micro-organisms in their digestive tract and that antibiotics encourage such synthesis. It is interesting to note that Frost, Overby & Spruth (1955) did not detect vitamin-sparing properties in several organic arsenicals though, as already mentioned, they may promote growth.

Practical feeding tests with pigs and poultry provide additional evidence for the sparing effect of antibiotics on vitamin B_{12} and, possibly, on unidentified growth factors. It is generally accepted that antibiotics have a greater growth-promoting action for pigs and poultry when added to all-vegetable rations likely to be deficient in vitamin B_{12} and other factors accompanying animal proteins (cf. review by Braude, Kon & Porter, 1953). Since vitamin B_{12} is frequently a product of bacterial fermentation, once again it is possible that antibiotics alter microbial activity in the gut, with resulting increased synthesis.

A number of workers have found increased stores of vitamin A in the livers of chicks (Burgess, Gluck, Brisson & Laughland, 1951; Coates, Harrison, Kon, Porter & Thompson, 1952) and pigs (Barber, Braude, Kon & Mitchell, 1952) receiving antibiotics. It is difficult to explain these observations on the grounds of altered microbial activity in the gut; a more likely possibility is that the antibiotic facilitates in some way the absorption of vitamin A or its formation from carotene.

Thus the addition of antibiotics to rations in which some of the vitamins are marginal in amount is likely to be of benefit to poultry and pigs. However, the beneficial effects of antibiotics are not entirely due to nutritional factors. Full discussion of their mode of action is outside the scope of this paper, but the problem is more fully reviewed elsewhere (Coates, 1953; Braude *et al.* 1953; Stokstad, 1954).

2. Factors Affecting Availability of Vitamins from the Diet

The need for increasing the amount of a vitamin in the diet of an animal may arise for several reasons, apart from such obvious ones as instability of the vitamin in a compounded diet. Thus, a vitamin may become less available to the animal in the presence of an antagonist; it may be present as a combined form from which the digestive enzymes cannot liberate the vitamin; further, the ordinary requirement may be increased by stress factors such as an abnormal balance of nutrients in the diet.

Several diseases or pathological conditions in practical animal nutrition have been traced to such conditioned vitamin deficiencies as instanced below.

a. Thiaminases

Enzymes that can split the thiamine molecule into two parts at the methylene bridge occur in raw fish, in certain ferns and in a few faecal bacteria (cf. Fijita, 1954).

Raw fish factor. Chastek paralysis was reported by Green, Carlson & Evans (1942) to occur in silver foxes fed on raw carp flesh; the factor responsible for this was identified by Scalock & Goodland, 1944, as a thiaminase. Meanwhile independent work in Japan had also shown that raw fish and many crustacea contain a thiaminase (Fijita & Numara, 1942). Diets containing raw fish have since been shown to produce thiamine deficiency in cats (Smith & Proutt, 1944) and chicks (Spitzer, Coombes, Elvehjem & Wisnicky, 1941).

Bracken factor. Air-dried bracken (*Pteris aquilina*), but not sun-dried or steamed bracken, causes in rats (Weswig, Freed & Haag, 1946) or horses (Evans, Evans & Roberts, 1951) signs of thiamine deficiency that are cured by administration of the vitamin. The active agent is a thiaminase behaving in many ways as does that of raw fish. Bracken poisoning also occurs in ruminants, though the signs are different and the condition is not cured by treatment with thiamine (Evans, Jones & Evans, 1950). The level of pyruvate in the blood of affected animals increases but little, and the levels of thiamine in the tissues are only slightly reduced (Moon & Raafat, 1951). Naftalin & Cushnie (1954) showed that the essential lesion in ruminants is extensive bone-marrow damage, affecting the megakaryocytes, the primitive white cells and the primitive red cells. Subsequently there are multiple haemorrhages, and bacterial invasion of the blood stream leads to a generalized non-specific bacteraemia. Thomas, Watkin, Evans & Evans (1955) have recently separated bracken extracts into a protein fraction containing thiaminase and a non-protein fraction free from thiaminase. The two fractions were given to sheep; those receiving the thiaminase showed a normal blood picture throughout the experiment, whereas the blood from those receiving the non-protein fraction showed changes similar to those found in the earlier stages of bracken poisoning. These results suggest that the factor toxic to the bone-marrow of ruminants is a dialysable substance distinct from thiaminase.

b. Bound Nicotinic Acid in Cereals

Microbiological and chemical evidence indicate that almost all the nicotinic acid in cereals such as maize and wheat is present in an alkali-labile "bound" form (cf. Kodicek, 1951b). This bound form of nicotinic acid does not appear to serve as a source of the vitamin for the nicotinic-acid-deficient rat, chick or pig (cf. Braude, Kon, Mitchell & Kodicek, 1955). The full biological activity of nicotinic acid for the rat can be released by hydrolysis with 0.5 N-sodium hydroxide (Chaudhuri & Kodicek, 1950; Kodicek, 1951a). The recent experiments of Braude *et al.* (1955), in which pigs were given diets containing maize before and after alkaline hydrolysis, show clearly that, whereas the unhydrolysed maize diet was pellagragenic, the hydrolysed diet allowed growth comparable with that of a control group receiving nicotinic acid. Until recently the most popular theory for the pellagragenic property of maize was that it contained a toxic factor or a factor interfering with the metabolism of nicotinic acid (see review by Chick, 1951).

Although in many parts of the world maize is used to a large extent as a component of rations for pigs and poultry, deficiency of nicotinic acid is uncommon in these animals. The published estimates of the nicotinic acid required by animals, based as they are on microbiological or chemical methods that include the biologically non-available form, may well require revision.

c. "Haemorrhagic Syndrome" in Poultry

The importance of vitamin K in poultry rations has become more apparent with the growth of the table poultry industry in the USA. Inadequacy of vitamin K leads to generalized haemorrhages throughout the tissues, and the occurrence of this "haemorrhagic syndrome" presents a serious commercial problem, since many carcasses have to be rejected because the flesh is stained. A similar condition has been reproduced experimentally by the use of certain coccidiostatic drugs such as arsanilic acid (Griminger, Fisher, Morrison, Snyder & Scott, 1953) and sulphaquinoxaline (Sweet, Romoser & Combs, 1954). One feature of the syndrome was prolonged clotting time of the blood, which could be restored to normal by supplements of vitamin K. Although Frost & Spruth (1955) failed to prolong clotting time in chicks by giving arsanilic acid, they reported that sulphaquinoxaline increased many times the chick's need for vitamin K. Contradictory evidence exists regarding the effect of dietary antibiotics on clotting time. Griminger *et al.* (1953) found that oxytetracycline delayed blood clotting, but this was not confirmed by Reynolds, Warden & Luther (1953). If, as is likely, the action of the drugs is to interfere with intestinal synthesis of vitamin K, small differences in conditions between different laboratories could account for the lack of agreement in results. On the other hand if, as work by Luckey *et al.* (1955) suggests, vitamin K is found in the tissues independently of bacterial synthesis, then these drugs might well interfere at some stage in its formation. In either event their effect is likely to be serious since, with modern methods of intensive management of poultry, precluding access to green food, the intake of vitamin K tends to be marginal.

The observation that the trypsin inhibitor found in raw soya beans has marked anticoagulant properties for human

blood prompted Balloun & Johnson (1953) to investigate the effect on blood-clotting time of feeding unheated soya meal, which would presumably contain the antitrypsin substance, to chicks. Although they found blood-clotting time to be significantly increased, it could not be restored to normal by addition of vitamin K to the diet, and the authors concluded that the antitrypsin substance inhibited blood coagulation not by virtue of vitamin K antagonism but possibly by interference at some point in the conversion of prothrombin to thrombin.

d. Hyperkeratosis (X Disease)

A new disease in cattle, of unknown cause, was described by Olafson (1947) and named X disease, or hyperkeratosis. Hyperkeratosis is most common in growing animals 3–12 months old, and the principal signs are the development of a thickened crusty skin, rapid loss of weight, anorexia and terminal diarrhoea; changes often occur in the mouth, and lesions consisting of papillary proliferations of the mucosa cause soreness and difficulty in eating. These signs are associated with a marked lowering of the concentration of vitamin A in the blood, suggestive of interference with vitamin A metabolism in the affected animal. The widespread incidence of the disease in certain parts of the USA, and the consequent heavy loss of animals, prompted a swift study of the nature of the causative agent. The disease was also reported in Germany (Wagener, 1951, 1952). Several groups of workers (cf. *Nutrition Reviews*, 1953) demonstrated that the typical signs of hyperkeratosis could be produced in cattle by the feeding of chlorinated naphthalenes, the more highly chlorinated derivatives being the more toxic. These chlorinated naphthalenes have many uses in industry (Sikes & Bridges, 1952; Sikes, Wise & Bridges, 1952). They are used in insecticides or as electrical insulators for wires and coils and as additives to lubricants, crank-case oils or cutting oils. Thus, in modern agricultural practice, cattle may have access to a number of sources of these compounds; for example, crank-case oil is used widely as an insecticide on cattle, and the lubricants used in the preparation of commercial feed pellets were shown to contain chlorinated naphthalenes.

From the nutritional standpoint the antivitamin A effect of chlorinated naphthalenes is of considerable interest. Since the concentration of carotene in the blood of affected animals remains relatively constant, whereas that of vitamin A falls rapidly, it is possible that the chlorinated naphthalenes interfere with the conversion of carotene to vitamin A (Hansel, McEntee & Olafson, 1951). However, Hoekstra, Hall & Phillips (1954) showed that the vitamin A in the plasma was depressed by large quantities of hyperkeratotic agents even when vitamin A itself was being administered at the same time. Although several explanations of the toxic action are possible, it seems likely that the toxic agent causes chemical destruction or inactivation of the vitamin A molecule, since Hove (1953) found that in cattle showing signs of X disease there was not only a decrease in vitamin A but also in the vitamin E concentration in the blood plasma. Thus the chlorinated naphthalenes may act through a pro-oxidant effect on unsaturated lipids and fat-soluble vitamins.

e. Rachitogenic Factors

Experiments with large quantities of yeast in pig rations as a protein source in the wartime shortage of other protein supplements led to the discovery by Braude, Kon & White (1943, 1944) of a rachitogenic factor in yeast. More recently the rachitogenic effect of green oats on sheep was reported by Ewer (1950) in England and by Grant (1951) in New Zealand. Weits (1952) also detected rachitogenic activity for rats in the fat extracted from hay. In all these instances the rachitogenic effect appears to be the result of vitamin D antagonism rather than direct interference with the metabolism of calcium and phosphorus, but there is no evidence that the rachitogenic principle is the same from all three sources. The nature of the rachitogenic factor in yeast has not yet been elucidated. Grant (1953) fractionated young green oats and came to the conclusion that the active rachitogenic factor was carotene; this was supported by Weits (1954) who, on fractionation of grass fat, found that the rachitogenic factor accompanied carotene. However, unpublished experiments at this Institute with rats and chicks have not confirmed the rachitogenic properties of β-carotene or vitamin A; this suggests that the final solution to this problem has not yet been reached. We have recently detected marked antivitamin D activity in raw pig's liver. This finding, together with the evidence quoted above, suggests that rachitogenic factors may be more widespread than has hitherto been recognized.

f. Oxidative Agents and Vitamin E

Vitamin E deficiency in animals is characterized by locomotor disturbances and general muscular dystrophy.

The dietary requirement for vitamin E cannot be assessed without reference to the other constituents of the diet, for it is abundantly clear that the presence of unsaturated fatty acids in the diet markedly increases the requirement (cf. Blaxter & Brown, 1952). Recent experiments by Blaxter and his colleagues have shown that the recommended, and widely practised, addition of cod-liver oil to a dried skim-milk diet for calves results in greatly increased incidence of muscular dystrophy (Blaxter, Wood & Macdonald, 1953). From their subsequent work (Blaxter, Brown & Macdonald, 1953) it is clear that the toxicity of cod-liver oil to the calf resides in the unsaturated fatty acid fraction.

Draper, James & Johnson (1952) produced experimental vitamin E deficiency in lambs by including in the diet tri-*o*-cresyl phosphate, shown by Meunier, Vinet & Jouanneteau (1949) to be a vitamin E antagonist. They identified this condition histologically and clinically with the widely distributed "stiff-lamb disease" described by Metzger & Hogan (1927).

Studies in vitamin E deficiency in poultry by Singsen and his colleagues have shown a direct correlation between the degree of unsaturation of fish oils used in chick diets and the percentage mortality due to encephalomalacia in the chicks receiving them (Singsen, Potter, Matterson, Bunnell, Kozeff & Jungherr, 1954). These workers observed that a low blood level of vitamin E did not itself result in encephalomalacia, but the addition of fish oil to the diet induced a high mortality from encephalomalacia that could be completely prevented by supplements of vitamin E (Singsen, Bunnell, Matterson, Kozeff & Jungherr, 1955). Chicks were also protected from encephalomalacia by addition of 0.025% diphenyl-*p*-phenylenediamine (DPPD) to the diet. These authors suggest that the function of vitamin E in preventing encephalomalacia is that of a general biological antioxidant, and that DPPD parallels some of the actions of vitamin E by virtue of its own

antioxidant properties. Other antioxidants such as methylene blue have been shown to have some of the antioxidant properties of vitamin E (see Moore in this number of the Bulletin, p. 44).

g. Availability of Vitamin B_{12}

Since vitamin B_{12} appears to be required by animals in only minute amounts, a large part of which will be obtained from synthesis in the gut either by coprophagy or possibly by direct absorption, a serious deficiency of vitamin B_{12} is unlikely to be encountered in practice, except perhaps in poultry kept intensively. In this system of management, when the birds are out of contact with their droppings, a dietary source of vitamin B_{12} is necessary. In its absence growth of young birds is retarded, and although adult hens are apparently unaffected by the deficiency, the eggs they produce do not hatch normally. The greatest embryonic mortality occurs towards the later stages of incubation, and the dead embryos show haemorrhagic patches throughout the tissues (Olcese, Couch, Quisenberry & Pearson, 1950; National Institute for Research in Dairying, 1953; Ferguson & Couch, 1954). It is of interest to note that the signs of vitamin B_{12} deficiency in animals do not usually include anaemia. Although blood changes have been reported in baby pigs (Neumann, Johnson & Thiersch, 1950) reared on artificial " milk " lacking vitamin B_{12}, the haematological picture was not identical with that encountered in pernicious anaemia in man.

Vitamin B_{12} deficiency may be exacerbated by dietary stress. A diet high in protein increases the animal's need for vitamin B_{12} (Hartman, Dryden & Cary, 1949; Yacowitz, Miller, Norris & Heuser, 1952). Frölich (1953) observed that the vitamin B_{12} requirement of chicks given diets containing soya varied with the quality of the meal, in that their need was greater with underheated soya meal. It is possible that some antagonism exists between soyin, a toxic principle occurring in raw soya, and vitamin B_{12}; it is, however, more likely that the trypsin inhibitor present in underheated soya prevented full utilization of methionine by interference with digestion of proteins and hence increased the need for vitamin B_{12}.

Other components of the diets may also influence the requirement for vitamin B_{12}. Thus, Spivey, Briggs & Ortiz (1954) precipitated signs of vitamin B_{12} deficiency in chicks by including 20% of lard in the diet, an observation which may be of some practical importance in view of the recent trend in the USA to dispose of surplus fat in rations for livestock.

Vitamin B_{12} is known to be present in many animal tissues in a bound form, and if such materials are to be used in diets as sources of vitamin B_{12} it is important to know how well they are utilized by the animal receiving them. We have evidence (Coates, Gregory, Harrison, Henry, Holdsworth & Kon, 1955) that the bound vitamin B_{12} from some animals is not fully available to other species. For instance, the vitamin B_{12} in sow's milk, which seemingly is available to the suckling piglet, is only very poorly utilized by the chick. Although the mechanism of binding is not yet fully understood, it is obviously a factor to be taken into account when compounding livestock rations.

3. Conclusions

Modern husbandry, aimed at the greatest production of meat, milk and eggs, imposes undue physiological stress on domesticated animals. Improvements in the quality of feeds and intensive methods of management have raised the growth-rate and performance of pigs and poultry to levels unheard of twenty years ago. In milk production, yields that were isolated records then are now commonplace. Such unnaturally rapid metabolic turnovers make special demands on enzyme systems in which vitamins take part. Man's knowledge of their function under such conditions and of their interconnexion with other nutrients lags well behind the vast amount of information about their chemical and biochemical properties and normal physiological role. It is only natural that workers should seek solution of some of the baffling problems in animal nutrition created by man's will and, all too often, by his ignorance. It has been our aim in this article to indicate these recent trends.

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ANTIVITAMINS, ANTIMETABOLITES AND CHEMOTHERAPY

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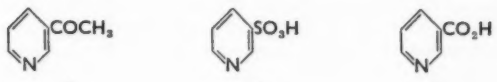
1 Antivitamins
  a Historical
  b The fat-soluble vitamins
  c The water-soluble vitamins
  d Effect on micro-organisms
  e Structural changes that convert vitamins into antivitamins
2 Antimetabolites other than antivitamins
  a Water, hydrogen ions and other inorganic ions
  b Amino acids, purines and pyrimidines
  c Miscellaneous
3 Chemotherapy
References

When a small detail of the molecular structure of a metabolite[1] is altered, some (or even all) of the biological activity of the original substance is usually lost. In some cases, the new substance is actually capable of antagonizing the biological action of the metabolite, and is then called an antimetabolite. The antivitamins are merely those antimetabolites that antagonize the action of vitamins. A number of antivitamins, such as the sulphonamides, *p*-aminosalicylic acid, pyrimethamine and A-methopterin, are powerful chemotherapeutic agents and are widely used in medicine.

1. Antivitamins

a. Historical

The first antivitamin was accidentally discovered in 1938. Woolley, Strong, Madden & Elvehjem, expecting 3-acetylpyridine (I) and pyridine-3-sulphonic acid (II) to have, at least qualitatively, the biological action of the vitamin, nicotinic acid (III), fed these analogues to dogs suffering from nicotinic acid deficiency. To their surprise, the condition was worsened. No clear picture of what was happening emerged until Woods (1940) demonstrated the reversal of the antibacterial action of sulphanilamide (IV) by *p*-aminobenzoic acid (V), and showed that this reversal depended upon the structural similarity of the two substances. It had long been known that enzymes are inhibited by substances which structurally resemble their substrates. Thus Wohl & Glimm (1910) found that amylase, the enzyme which converts starch to maltose, is inhibited by glucose. Likewise, malonic acid (VI) competitively inhibits the enzyme succinic dehydrogenase by displacing the normal substrate, succinic acid (VII), from this enzyme, which normally converts (VII) to fumaric acid (Quastel & Wooldridge, 1927). The importance of Woods' discovery lies in his recognition that this phenomenon, well enough established in biochemistry, was also applicable to bacterial nutrition and hence to chemotherapy.



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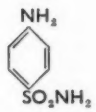

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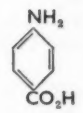


V

$HO_2C.CH_2.CO_2H$

VI

$HO_2C.CH_2.CH_2.CO_2H$

VII

VIII

In 1940, Fildes suggested that useful chemotherapeutic agents could be produced by altering the structure of the vitamins or metabolites just sufficiently to obtain an antagonist. McIlwain (1940) showed that pyridine-3-sulphonic acid (II) inhibited several bacteria which required an external source of nicotinic acid (III) for growth, and that growth was restored when an excess of nicotinic acid was supplied. Then, American, German and British workers showed that pantoyltaurine (VIII, R = SO_3H) competitively inhibited the growth-promoting effect of pantothenic acid (VIII, R = CO_2H) in several types of bacteria (Snell, 1941; Kuhn, Wieland & Möller, 1941; McIlwain, 1942). The seed had now been sown: in the next few years a great harvest was reaped.

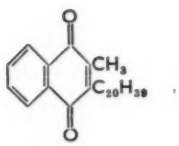
The antivitamins will now be discussed in detail; in sections 1b and 1c, the word " vitamin " will be used only with reference to animals. Almost every known antivitamin can be mentioned, because so few examples are known. (Perhaps the difficulties of synthetic work have prevented chemists from supplying other analogues in the rather large quantities required for animal experiments.) In section 1d, the results with micro-organisms are reviewed: many more " antivitamins " have been recognized in this field, and it will not be possible to mention each by name.

b. The Fat-Soluble Vitamins

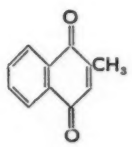
No potent antagonists of vitamins A, D or E are known, but several anti-vitamins K have been discovered. Vitamin K_1 (IX), believed to be essential for the formation of prothrombin in blood, is synthesized by bacteria in the intestine, and this is the sole human source of the K vitamins. The $C_{20}H_{39}$ (phytyl) group is a fatty side-chain not essential for vitamin K activity, for it may be replaced by hydrogen as in menaphthone (X), giving a substance of equal biological activity, which is much used in medicine in cases of vitamin K deficiency.

[1] A naturally occurring substance, of low molecular weight, playing an important part in metabolic processes, e.g. coenzymes, vitamins, hormones, minerals, and substrates.

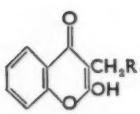
Antibacterial substances, such as sulphonamides, exert anti-vitamin K activity simply by killing the intestinal bacteria which synthesize this vitamin. Substances with certain types of fatty side-chain, notably glycerides of dihydroxystearic acid, prevent the bacteria from synthesizing vitamin K_1, presumably by blocking the responsible enzyme (Nightingale, Lockhart & Harris, 1947). This hypothesis implies that the precursor of the $C_{20}H_{39}$ fatty side-chain of K_1 is displaced from the enzyme normally synthesizing this vitamin which is consequently not formed. Some types of antivitamin act through displacement of the vitamin from an enzyme or other receptor. Such antivitamins depend upon considerable structural similarity to the vitamins that they are displacing. Such an effect in the vitamin K series is shown by dicoumarol (methylene-bis-3-(4-hydroxycoumarin)) (XIa), isolated in 1941 from particular clover crops, which caused haemorrhagic disease in cattle. The anticoagulant effect of this substance is countered by vitamin K (Overman, Stahmann, Sullivan, Huebner, Campbell & Link, 1942). Extensive studies of structure-action relationships showed that dicoumarol is only one of many hydroxycoumarins (XI) substituted in the 3-position which have anti-vitamin K properties (Overman, Stahmann, Huebner, Sullivan, Spero, Doherty, Ikowa, Graf, Roseman & Link, 1944).



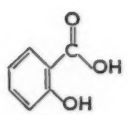
IX



X



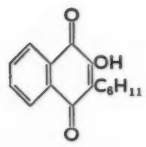
XI



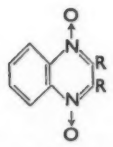
XII

(a) In dicoumarol, R is another hydroxycoumarin nucleus

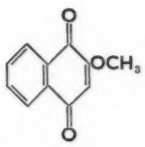
Although formula (XI) has, on sound chemical grounds, been written in the tautomeric form most resembling vitamin K, there remain many points of difference between the two molecules. Yet even salicylic acid (XII) has anticoagulant action, reversible by vitamin K. Thus, we have an early indication of the difficulties met in devising antivitamins, because we can hardly know in advance which parts of the vitamin molecule must be repeated in the antivitamin, and which parts may be omitted. However, anti-K compounds that are closer in structure to the vitamin are known. 2-*cyclo*Hexyl-3-hydroxynaphthoquinone (XIII) has typical anticoagulant activity reversed by vitamin K (Smith, 1947). Some quinoxaline-*N*-oxides (XIV) and the related phenazine antibiotic, iodinin, have a structural relationship to vitamin K, and their antibacterial action is reversed by this vitamin (McIlwain, 1943). This was the first antagonism of a metabolite by an antibiotic to be recorded. 2-Methoxynaphthoquinone (XV), an antibiotic occurring in the balsam, a garden plant, has some anti-vitamin K activity (Molho, Moraux & Meunier, 1948).



XIII



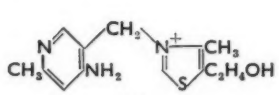
XIV



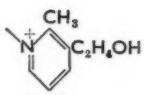
XV

c. The Water-Soluble Vitamins

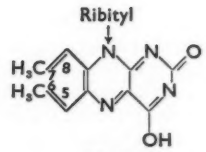
Thiamine (aneurin) (XVI) occurs in animal tissues as a pyrophosphoric ester known as cocarboxylase. The enzyme thiaminase, which decomposes thiamine, causes deprivation symptoms in animals fed on raw fish in which this enzyme occurs. A different kind of antivitamin, one that displaces thiamine from its phosphorylating enzyme, is pyrithiamine, in which the thiazole portion of thiamine (XVI) is replaced by an isosteric pyridine ring (XVII). The first samples prepared, although impure, produced a characteristic thiamine deficiency in mice, which could be cured by roughly one-fortieth of a molecular equivalent of thiamine (Woolley & White, 1943a, 1943b). When pyrithiamine was finally obtained pure it was found that only ten molecules were required to antagonize one of thiamine (Wilson & Harris, 1949; Woolley, 1950). Another, but less potent, antithiamine is oxythiamine, in which the amino group of the pyrimidine ring of thiamine is replaced by hydroxyl. This was first made by Bergel & Todd (1937), and was shown to antagonize thiamine (Frohman & Day, 1949; Daniel & Norris, 1949; Eusebi & Cerecedo, 1949). It causes death, but without the symptoms of polyneuritis produced by pyrithiamine, and this may provide a clue as to the specificity of the two halves of the molecule of thiamine in influencing distribution to different target areas. Whereas oxythiamine prevents the formation of cocarboxylase, oxythiamine diphosphate appears to prevent the utilization of this coenzyme (Eusebi & Cerecedo, 1950).



XVI



XVII



XVIII

Riboflavin (XVIII) retains some vitamin activity when either methyl group (but not both) is deleted. An antivitamin effect had been described in rats treated with an isomeride in which the 7-methyl had been shifted to the 5-position (Emerson & Tishler, 1944), but the authors later failed to confirm this (Woolley, 1952). Several analogues in which the ribityl group is replaced by other sugar alcohols have weak antivitamin action (Kuhn, Weygand & Möller, 1943), and apparently the most powerful of these is the galactityl analogue (Emerson, Wurtz & Johnson, 1945). Changing the pyrimidine

ring (on extreme right of XVIII) to a 2 : 4-dinitrobenzene ring gave a phenazine derivative which showed a powerful anti-riboflavin action in mice (Woolley, 1944).

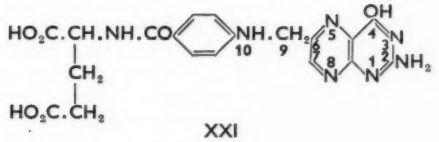
The B_6 group of vitamins occurs in the three forms shown in (XIX). The analogue "desoxypyridoxine" (XIX, R = CH_3) produces signs of B_6 deficiency in chicks and rats, reversible by pyridoxine (Ott, 1946; Emerson, 1947). Experiments with enzymes suggest that this analogue is inactive until it is phosphorylated in the body, and that the derivative so formed prevents the attachment of pyridoxal phosphate to the apoenzyme of the various amino-acid decarboxylases (Beiler & Martin, 1947; Umbreit & Gunsalus, 1949; Umbreit & Waddell, 1949). Deoxypyridoxine, given in a 1 : 1 ratio to the B_6 naturally present in the body, caused B_6 deficiency in man, and this was cured by pyridoxine in three days (Mueller & Vilter, 1950). "Methoxypyridoxine" (XIX, R = CH_2OCH_3) shows antivitamin activity in chicks (Ott, 1947), but rats and mice degrade it to pyridoxine. The antituberculosis drug, isoniazid, causes some signs of pyridoxal deficiency in man, apparently by converting this aldehyde into its *iso*nicotinyl hydrazone (Biehl & Vilter, 1954).

Nicotinic acid (III) has already been mentioned in section 1a. In mammals it occurs as the amide (XX) which is further elaborated to give the two coenzymes known as diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN). It was while working with nicotinamide that McIlwain (1940) invented "inhibition analysis", some examples of which have already been given. McIlwain noted that pyridine-3-sulphonic acid (II) inhibited growth of the bacterium *Proteus* when promoted by DPN, but not when promoted by nicotinamide, and deduced that the latter was used by *Proteus* for some important purpose other than the synthesis of DPN. Pyridine-3-sulphonic acid does not produce nicotinamide deficiency in mice (Woolley & White, 1943a, 1943b), but 3-acetylpyridine (I) does, and either nicotinic acid or nicotinamide can reverse it (Woolley, 1945).

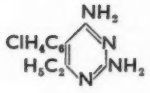
XIX

XX

Pyridoxine (R = CH_2OH)
Pyridoxal (R = CHO)
Pyridoxamine (R = CH_2NH_2)



XXI



XXII

Recently, the principal target of antivitamin research has been the folic acid family, the best-known member of which is pteroylglutamic acid (PGA) (XXI) which is built up of glutamic acid, *p*-aminobenzoic acid and a tri-substituted pteridine. In the living cell, folic acid seems to be present as the 5 : 6 : 7 : 8-tetrahydro form, also as the "citrovorum factor" (a formyl derivative of the latter), which is a component of a biochemical complex which includes adenosine triphosphate (Greenberg, 1954). Mammalian cells require the citrovorum factor during mitosis to carry metaphase on to anaphase (Jacobson & Webb, 1952). As with vitamin K, mammals seem to be dependent on intestinal bacteria for their supplies of the folic acids, and deficiencies can be caused by intestinal antiseptics. Of these, the sulphonamides are specific because their bacteriostatic action arises from their suppression of synthesis of folic acids (see section 1a).

Prevention of the utilization of folic acid was first demonstrated with the 7-methyl derivative of PGA (regrettably used in an impure state). Rats fed with this antivitamin showed haematological changes typical of folic acid deficiency, and these were reversed when PGA was given (Martin, Tolman & Moss, 1947; Franklin, Stokstad, Belt & Jukes, 1947). Aminopterin (an analogue of PGA in which the 4–OH group is replaced by —NH_2) causes similar changes, but these are not reversed by PGA, although reversal occurs with the citrovorum factor (Nichol & Welch, 1950; Broquist, Stokstad & Jukes, 1950): thus interference occurs at a later stage than with the earlier antivitamin. Other active substances have been made from PGA by methylating $N_{(10)}$, by replacing the glutamic acid by aspartic acid, and by effecting one (or both) of these changes in conjunction with the above-mentioned amination in the 4-position.

Of the remaining water-soluble vitamins (biotin, pantothenic acid, inositol, cyanocobalamin or B_{12}, and ascorbic acid), it is doubtful whether any structural analogue is known capable of producing a pronounced antivitamin effect in animals. Avidin, a protein in egg white, which has a great affinity for biotin, is the only well-established antivitamin of the non-displacement type.

d. Effect on Micro-Organisms

The metabolism of bacteria and of most of the other unicellular organisms involves many of the vitamins required by vertebrates. Some micro-organisms meet their own vitamin requirements by syntheses; in such cases, antimetabolites which prevent some of the syntheses are known, sulphonamides being a well-known example. In other cases, vitamins have to be obtained from the external environment, a situation analogous to that existing in man.

Some antivitamins, such as 3-acetylpyridine (I), are active in vertebrates but not in bacteria. Conversely others, such as pyridine-3-sulphonic acid (II), are active in bacteria but not in vertebrates. However, usually a substance which shows antivitamin action in animals also shows it in bacteria. The reverse is not true: of all the many analogues of pantothenic acid effective against bacteria and protozoa, it is doubtful if more than one causes even the slightest deficiency in vertebrates. In other cases, the difficulties of synthesis have prevented sufficient material being accumulated for tests in animals which naturally require much more than bacteria. It is not possible to mention here all the antivitamins active in micro-organisms, but most of them are covered by the monographs of Woolley (1952) and Martin (1951).

e. Structural Changes that Convert Vitamins into Antivitamins

These changes must not be too small, or the substance will retain vitamin activity. Indeed, a change may be quite large without loss of vitamin activity if it takes place in a part of the molecule which is not intimately concerned in the anchoring or chemical activity of the vitamin (see vitamin K, under section 1b, and compare sulphadiazine with *p*-aminobenzoic acid). Unfortunately, the experimenter can learn which parts of a vitamin are unessential only by the slow process of removing atoms or groups from the molecule, one at a time.

The first effective changes were from the carboxylic acid (or carboxamide) group to the sulphonic acid (or sulphonamide) group, or to the ketonic group (see under section 1a). Isosteric changes of —CH=CH— to —S—, and vice versa, have often proved effective (cf. pyrithiamine). The exchange of OH for NH_2, and vice versa, have been mentioned above, and methyl groups have effectively been replaced by chlorine atoms, or shifted to new positions in the molecule, or introduced in place of hydrogen atoms. Aliphatic side-chains (e.g. ribityl or glutamyl) can be replaced by homologues or isomers. Hydroxyl or amino groups may be replaced by hydrogen, and hydrogen (see below) may be replaced by fluorine. Sulphur atoms may be replaced by oxygen, or else raised to a higher valence by combination with oxygen (as in the biotin analogues). Other variants are known, and doubtless many more will be found. However, it must be made clear that a structural change effective for one vitamin has not always proved effective for another.

2. Antimetabolites other than Antivitamins

a. Water, Hydrogen Ions and other Inorganic Ions

Water and the inorganic ions are the most fundamental substances taking part in metabolic processes and as prone as any others to the blocking action of suitably constituted analogues. Ethanol, for example, may be looked upon as an antimetabolite for water. Like water, it has strong hydrogen-bonding properties and it penetrates the tissues by ways ordinarily reserved for water (Albert, 1951b).

The presence of a hydrogen ion on a particular receptor (acid or amine) allows of rapidly reversible exchanges with other metabolically important cations. But drugs that (i) are cations and that (ii) also have a structure that specifically fits the area surrounding such a receptor can force off these hydrogen ions in spite of a high hydrogen-ion concentration in the solution surrounding the receptor. The action of the acridine antibacterials has been explained along these lines (Albert, 1951a), and it is shown that similar antibacterial activity may be expected from any organic cation having a flat area of 38 sq. Å, or more. It is reasonable to expect the ultimate mode of action of other cationic substances (including the alkaloids, local anaesthetics and the nitrogenous hormones) to be similar, each type of drug action corresponding to a different design surrounding the positively charged atom. It should be noted that in these cases the design would be not flat but three-dimensional.

The antagonism of calcium and potassium cations in the heart, and of calcium and magnesium in muscle, has long been known. Similar antagonisms between heavy metals have been discovered more recently (summarized by Albert, 1951b). Antagonism between anions is typified by that between cyanate and nitrite in *Nitrobacter* (Lees & Simpson, 1955), and the use of bromides as sedatives is accompanied by the displacement of Cl^- by Br^-.

Special (non-antibacterial) sulphonamides have been devised, the anions of which compete with the bicarbonate ion for a place on the enzyme carbonic anhydrase in the renal tubules (Roblin & Clapp, 1950). Blocking this enzyme causes bicarbonate ions to accumulate and become excreted with sodium ions, thus causing a therapeutically useful diuresis. Acetazoleamide (Diamox), which is used for this purpose, is 2-acetamido-1 : 3 : 4-thiadiazole-5-sulphonamide.

b. Amino Acids, Purines and Pyrimidines

The natural amino acids are antagonists of one another. For example, the growth of *Bacillus anthracis* is accelerated if valine and leucine are added together, but retarded if only one of these acids, which are structurally similar, is added (Gladstone, 1939). The use of artificial analogues dates from the accidental discovery that ethionine is an antagonist of methionine in rats (Dyer, 1938). Artificial analogues of almost every amino acid are now available. When it was discovered that flour treated with nitrogen trichloride produced fits in dogs (Mellanby, 1946), the toxic agent was found to be methionine sulphoximine (Bentley, McDermott & Whitehead, 1950). Curiously enough, although this is derived from methionine, for which it is an antagonist, it is an even better antagonist of glutamine (Heathcote & Pace, 1950). The study of antimetabolites as complex as polypeptides, and even as proteins, has begun.

Structural analogues of adenine, guanine, thymine and cytosine are known, which have strong inhibitory action in certain systems; and useful applications to chemotherapy have been made (see section 3). Because nucleic acids play such an important part in anabolic processes and hence hold the key to selective toxicity, it seems likely that many more analogues will be made, especially as it has recently become possible to synthesize unnatural dinucleotides (Michelson & Todd, 1955). Acceleration of the growth of a lactobacillus by deoxyribonucleic acid (DNA) and retardation by ribonucleic acid (RNA) represents the extreme in molecular complexity in this series (Skeggs, Spizizen & Wright, 1950).

c. Miscellaneous

Space does not permit even listing by name the innumerable other antimetabolites that are now known; the reader is referred to the standard books (Woolley, 1952; Martin, 1951). Many hormones such as testosterone, adrenaline, thyroxine, acetylcholine, serotonin, and histamine now have antagonists, some of them of high specificity. Other antagonists have been discovered in investigating carbohydrate metabolism (see also section 1a), and of these the most remarkable is fluoroacetic acid, a naturally occurring substance poisonous to cattle. This substance is accepted by the cell as a substitute for acetic acid and is put through the usual stages of the Krebs cycle without injury to the cell until it eventually becomes fluorocitric acid. At this stage it competes against citric acid, and death quickly ensues (Liébecq & Peters, 1949). This process is known as lethal synthesis (Peters, Wakelin & Buffa, 1953).

3. Chemotherapy

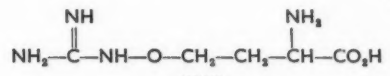
Stimulated by Fildes' suggestions (Fildes, 1940), many attempts have been made to obtain effective drugs by the synthesis of structural analogues of vital metabolites. The

number of successes has been small for various reasons. Sometimes the analogue was found to be broken down by the bacterium to the metabolite (e.g. 3-acetylpyridine to nicotinic acid), sometimes it was not possible to administer an adequate dose on grounds of expense or of speedy elimination, and in the majority of cases the analogue was as toxic to the host as to the parasite. Nevertheless, a few outstanding successes have been achieved.

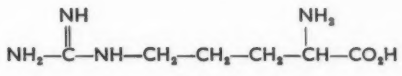
Some of the most striking results have been obtained in malaria. Phenylpantothenone (VIII, R = COC_6H_5), an antagonist of pantothenic acid, is as effective in man as quinine (Mead, Rapport, Senear, Maynard & Koepfli, 1946). A more powerful antimalarial drug, pyrimethamine (Daraprim) (XXII), has since been evolved on equally rational lines, after it was observed that several substances containing the 2 : 4-diaminopyrimidine nucleus were antagonists of pteroylglutamic acid, yet of low toxicity to mammals (Hitchings, 1952). The 2 : 4-diaminopteridines are more toxic to man, but have been successful in delaying death in one form of cancer: the leukaemia of childhood. For this purpose A-methopterin (the 10-methyl derivative of Aminopterin; see section 1c) is considered most satisfactory, although the over-active marrow cells become resistant to it in a few months' time (Burchenal, Waring & Hutchinson, 1951). Even 2 : 4-diaminopteridine itself is strongly antileukaemic in man (Jacobson, personal communication, 1955).

Of particular interest for the future of chemotherapy are those analogues that are built by the cell into its metabolites, which do not then function satisfactorily (see also lethal synthesis, under section 2c). For example, the antituberculosis drug, *p*-aminosalicylic acid, is incorporated into the folic acid of some organisms (Wacker, Grisebach, Trebst, Ebert & Weygand, 1954). 8-Azaguanine (an analogue of guanine) is incorporated into the nucleic acids of tumours (Mitchell, Skipper & Bennett, 1950); tobacco mosaic virus builds 8-azaguanine (Matthews, 1954) and thiouracil (Jeener & Rosseels, 1953) into its ribonucleic acid. 5-Bromouracil replaces thymine in *Streptococcus faecalis* (Weygand, Wacker & Dellweg, 1952); and 6-mercaptopurine, an inhibitor of certain tumours, is incorporated into both RNA and DNA in mice (Elion, Bieber & Hitchings, 1954).

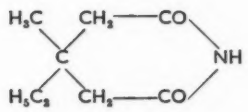
In the virus problem, examples of direct approaches are seen (i) in the inhibition of poliomyelitis in mice by oxythiamine (see section 1c), a small but definite susceptibility to thiamine deficiency between host and virus being claimed (Jones, Foster & Henle, 1948); and (ii) in the inhibition of multiplication of influenza virus by the amino acid canavanine (XXIII), and its reversal by arginine (XXIV) (Pilcher, Soike, Smith, Trosper & Folston, 1955).



XXIII



XXIV



XXV

There can be little doubt that, as knowledge of comparative biochemistry increases and the differences in intermediary metabolism are brought to light, many rational applications of antagonism in chemotherapy can be made.

In conclusion, attention is drawn to the particular success with which antagonists to accidental poisoning are now rationally devised: e.g. BAL, i.e., British anti-lewisite (2 : 3-dimercaptopropanol) against arsenical drugs (Peters, Stocken & Thompson, 1945); nalorphine (allyl-normorphine) against morphine (Unna, 1943); ethylenediaminetetraacetic acid against lead (Giles, Moore & Still, 1955); and β : β-methylethylglutarimide (XXV) against barbiturates (Shaw, Simon, Cass, Shulman, Anster & Nelson, 1954).

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EFFECT OF PROCESSING ON THE VITAMIN CONTENT OF FOODS

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Studies of the nutritive value of processed foods have been mainly concerned with loss of vitamins. Less consideration has been given to changes that processing may cause in proteins, fats, carbohydrates or mineral elements, present evidence suggesting that the changes in these constituents are small compared with those of other nutrients. In considering the effects of any process on the nutritive value of food-stuffs, it has to be appreciated that factors such as variety, conditions of growth, and the maturity of the raw material may outweigh the losses of nutrients during processing; this is especially true of vegetable foods. Of equal importance, with vegetables, are the losses which may result from careless handling during collection and transport. Unlike most foods of animal origin, fruit and vegetable tissues are living even after being harvested; and the processes of respiration and transpiration proceed in them at a rate which is determined by factors like temperature, exposure to conditions which cause wilting, or damage by bruising, cutting or freezing.

A survey of the loss of nutrients from these causes in fruit and vegetables has been published (Mapson, 1952). The main conclusions were: that the loss of nutritive value is small if (i) the time between harvesting and distribution is not unduly prolonged; (ii) the temperature during transit is kept low enough to reduce enzymic change to a minimum without freezing the cell sap; and (iii) reasonable care is taken in handling. These conditions are likely to be realized in commercial practice, since nearness to the factory and rapid handling of the raw material are both essential to efficient production.

The objectives of modern food preservation are (i) the suppression of micro-organisms which would otherwise cause spoilage; (ii) the preservation of an attractive quality (colour, flavour and texture); and (iii) the maintenance in the processed product of the nutritive value of the fresh food. These objectives are achieved by (i) increasing the osmotic pressure of the liquid phase of the food, thereby rendering it unsuitable as a medium for the growth of micro-organisms; (ii) sterilization by heat; (iii) refrigeration; and (iv) the addition of chemical preservatives. The first three of these methods are exemplified in dehydration, canning and freezing, respectively, and they account for the major proportion of our preserved food.

Loss of nutrients in modern methods of processing may occur by enzymic destruction, chemical oxidation, and extraction. Nutrients vary in their susceptibility to enzymic or chemical oxidation. Thus, ascorbic acid is particularly susceptible to both enzymic and chemical oxidation, and its high solubility in water renders it easy to extract from tissues. More than any other nutrient, it reflects deterioration in both quality and nutritive value, hence a fall in the ascorbic acid content of the tissue is a very good objective index, perhaps the best we have, for revealing whether the tissue has been subjected to stress. It is generally true for vegetable tissues that, if the ascorbic acid content has been well maintained during processing, it is unlikely that any serious loss of other nutrients will have occurred.

1. Vegetables

The foods in which loss of nutritive value is most likely to be serious are fruits and vegetables. Nutrients, especially ascorbic acid and carotene, may be lost during the cleansing and preparation of vegetables, the scalding, and the process of drying, canning or freezing. The first two operations are preliminaries to drying, freezing and canning alike.

a. Losses during Cleansing and Preparation

During these operations, which include trimming and shredding, there is a danger that damage to the tissues, especially to those of green leafy vegetables, may result in a loss of ascorbic acid, and—to a smaller extent—of carotene, by enzymic oxidation. These losses will be proportional to the cutting and bruising (Allen, Barker & Mapson, 1943). Some small loss of water-soluble constituents may also occur during washing of cut surfaces, but the mere soaking of intact green leafy vegetables does not involve any significant diffusion of water-soluble constituents.

b. Losses during Scalding and Subsequent Cooling

It has long been a general practice to scald or "blanch" vegetables for a few minutes in boiling water or steam before processing. This scalding serves as an additional cleansing operation, improves the physical properties and the culinary quality of the final product and, most important of all, inactivates the enzymes which otherwise would cause the development of off-flavour, and the rapid loss of carotene (provitamin A) and ascorbic acid. Thus, Zscheile, Beadle & Kraybill (1943) found that frozen unscalded spinach, asparagus, peas, snap beans and lima beans lost a large proportion of their carotene content in 6–12 months' storage at −4° F.[1], whereas the properly scalded vegetables lost relatively little. Similarly, Farrell & Fellers (1942) found that, during storage, frozen unscalded green beans lost practically all of their ascorbic acid and a large proportion of their thiamine and riboflavin, whereas properly blanched beans lost relatively little of these vitamins.

Losses of nutritive constituents during scalding occur for two reasons: oxidation and extraction (leaching), the second being by far the more important. The vegetables are scalded

[1] t° F. = ⅝ (t − 32)° C.—Ed.

in boiling water for a short period (2–3 min.), or in steam. During the immersion of the raw vegetables in the scalding liquor there is rapid diffusion into it of all their water-soluble constituents, and in single scalds the loss may be quite high. Adam, Horner & Stanworth (1942) studied the losses of vitamin C, minerals and proteins during the scalding of peas, beans and carrots, potatoes, swedes, and sprouts. They found that small units of large surface area lost 19–35% of their sugars, 14–22% of their proteins, 17–30% of their mineral salts and 32–50% of their vitamin C, while the larger roots and starchy seeds lost 10–21% of their sugars, 2–8% of their protein, 8–16% of their minerals, and 22–33% of their vitamin C. Such losses naturally depend on the duration and temperature of scalding. Better retention of water-soluble nutrients (ascorbic acid, thiamine, nicotinic acid and riboflavin) has generally been obtained with high-temperature short-time scalds than with long-time scalds at either high or low temperature (Wagner, Strong & Elvehjem, 1947; Guerrant, Varich, Fordig, Ellenberger, Stern & Coonen, 1947). To reduce loss by leaching and oxidation, the scalding process should therefore be as short as possible, sufficient only to inactivate enzymes.

The adoption of the technique of serial scalding—i.e., the passage of repeated batches of fresh vegetables through the same scalding liquor—minimizes the loss (Allen *et al.* 1943) because the solutes extracted from the vegetables in the early scalds reach such a concentration in the scalding liquor that diffusion of solutes from later batches is prevented. Theoretically, a condition should be reached in which the concentration of solutes in the scalding liquor equals that in the fresh vegetables—then no further leaching would occur. In large-scale practice this state is never attained, for during scalding there is always a loss of scalding liquor by overflow from the scalder, as a result of the condensation of steam and the carry-over of water with the washed vegetable. Furthermore, with substances like ascorbic acid, some oxidation always takes place during scalding, which also reduces the concentration of the vitamin in the liquor. To minimize the oxidation of ascorbic acid during scalding and drying, sodium sulphite has been added to the scalding liquor as an antioxidant. In a laboratory trial of this practice, cabbage after serial scalding retained 94% of the ascorbic acid of the raw material, whereas, in conditions of manufacturing, the retention had fallen to 66%; the corresponding figures for potato were 82% and 63% respectively (Allen & Mapson, 1947; Wager, Tomkins, Brightwell, Allen & Mapson, 1945).

In some factories steam scalding is used instead of water scalding. This causes less loss by leaching, but losses due to oxidation are liable to be greater. From experiments on cabbage, it has been found that the losses of a substance like ascorbic acid are of the same order whether steam scalding or serial water scalding is adopted.

To avoid undue loss of ascorbic acid, carotene, and other readily oxidizable nutrients, the vegetable should be cooled as rapidly as possible after scalding; this may be done either by spraying with cold water, or by immersion in cold water. It is better, however, to cool rapidly in a blast of cold air, thus avoiding any further loss by leaching. In one experiment, cabbage which had been serially scalded retained 84% of the ascorbic acid of the fresh vegetable but, after cooling by immersion in water, the retention fell to 56%, the loss being due to leaching of the vitamin into the cooling liquid (Allen & Mapson, unpublished data). The over-all losses observed by American workers during the scalding operations of various vegetables ranged from 13% to 60% for ascorbic acid, 2% to 30% for thiamine, 5% to 40% for riboflavin, and 0.25% for carotene (Cameron & Esty, 1950).

c. Losses during Freezing, Drying and Canning

It is generally held that the losses of nutrients in these processes are small compared with those in scalding. There is no evidence that nutrients are lost when vegetables are frozen. It is probably true that no loss of nutrients occurs from fruits during freezing, although some loss of ascorbic acid has been reported when strawberries and raspberries are frozen in syrup; but this may have been due to the solution of the nutrients in the ice-crystals which separated out (Mayfield & Richardson, 1943). Other workers have reported little or no loss of this vitamin in fruits or fruit juices during freezing.

The loss of nutritive value during drying or canning is due to destruction, usually by oxidation. With freeze-drying no loss of ascorbic acid from peas was observed (Barker, Gane & Mapson, 1947), but, in hot-air drying, the temperature of the material is important if quality and vitamin content are to be maintained. It is possible to commence drying cabbage with air at 90° C. only because the temperature of the wet vegetable is, through evaporation, much lower than that of the air. In the later stages the air temperature must be diminished to 60° C. if loss of ascorbic acid is to be minimized (Allen *et al.* 1943). In large-scale factory production, between 10% and 14% of ascorbic acid was lost during these operations.

During canning, the loss of nutrients is usually small because, although temperatures of 100°–120° C. are customary, the low solubility of oxygen in water at these temperatures reduces oxidation. There is some evidence that thiamine may be more adversely affected by this heat treatment than other vitamins, especially in vegetables where the pH is near neutrality (Wagner *et al.* 1947). A review of the over-all loss of nutrients from fruit and vegetable foods on canning was given by Cameron & Esty (1950), and it has since been supplemented by Teply, Derse, Krieger & Elvehjem (1953). These results show that the loss of any particular nutrient varies with the type of fruit or vegetable processed.

On an average, canned vegetables retain 60% of the water-soluble nutrients in the raw material; but these are distributed between the solid and liquid contents of the cans and, if only the solids are eaten, the percentage actually consumed will be reduced to about 40. With fruits the retention is higher than this, mainly because no scalding treatment is necessary before canning, and secondly because the more labile vitamins (ascorbic acid and thiamine) are more stable in acid foods.

2. Meat and Fish

Meat and fish, though valuable sources of members of the vitamin B group, are chiefly important as sources of good protein and for increasing the palatability of the diet. In nutritive value, the flesh of fatty fish is superior to meat, and the flesh of white (non-fatty) fish is at least equal to it. Both types are excellent sources of magnesium and phosphorus, and contain vitamins of the B group in concentrations similar to those in lean meat. In addition, the tissues of fatty fish are rich in vitamin D and contain significant amounts of vitamin A.

Both meat and fish are preserved by freezing, and meat may be preserved for fairly short periods by storage at −2° C. As with fruit and vegetables, there is no evidence of any significant loss of nutrients during freezing, any loss being due to the formation of exudate (drip) during thawing; thus, when beef is thawed, 8–14% of all the vitamins of the B group may be lost in the exudate (Pearson *et al.* 1950).

Meat was successfully preserved by dehydration on a large scale during the Second World War. The trimmed meat was first cooked and minced, care being taken to return the cooking liquor to the minced meat before drying, to conserve water-soluble nutrients. The biological value of meat dried at 80° C. equalled that of freshly cooked meat, but a serious decline in the biological value of meat protein occurred when the temperature rose to 125° C. during drying (Cruickshank, Kodicek & Wang, 1946).

During the cooking of meat, losses of thiamine also occur, amounting to one-third of that present in the fresh material (Sharp, 1953; Rice & Robinson, 1944b), and these are paralleled by losses of pantothenic acid, pyridoxine, and folic acid; riboflavin and nicotinic acid are more stable, and their loss seldom exceeds 10%. Thiamine can also be lost during drying: for instance, at 70° C. there is a loss of 18% of this vitamin, increasing to 27% at 100° C. (Sharp, 1953).

In canning, as in cooking, the temperature and duration of the process govern the losses of vitamins of the B group. Nicotinic acid and riboflavin are considered to be stable during commercial canning procedures, but losses of thiamine (30–60%) and of pantothenic acid (24%) have been reported (Reedman & Buckby, 1943; Rice & Robinson, 1944a; Greenwood, Kraybill, Feasten & Jackson, 1944). With the methods of home canning, even greater losses are encountered, when the temperatures are higher than those used in commercial practice (Cover, Dalsaver & Hays, 1949).

Our knowledge of the changes in the nutritive values of fish flesh during processing is more scanty. Losses of the B vitamins are probably of the same order as those in meat, except that thiamine appears to be more labile (Lopez-Matas & Fellers, 1948; Pyke & Wright, 1941): this may be due to the occurrence in fish of thiaminase, an enzyme which destroys it. Lunde (1939) found no loss of vitamin in canning fish, provided that all air was removed; but a survey by Bacharach and his co-workers (Bacharach, Cruickshank, Henry, Kon, Lovern, Moore & Morton, 1942) indicated that there must normally be some loss, because there is always less vitamin A in canned than in fresh herring. Changes in vitamin content during the smoking of fish have been studied by Lunde (1937), by Lunde, Aschekong & Kringstad (1937), by Pyke (1939), and by McVicar & Berryman (1942): nicotinic acid, riboflavin and vitamin D were unaffected but there was some loss of vitamin A.

3. Eggs

Eggs are preserved by (i) storage at 0° C. in a humidified atmosphere, sometimes enriched with carbon dioxide; (ii) treatment of the shell egg with sealing agents; (iii) freezing whole liquid egg; and (iv) drying whole liquid egg. Changes of nutritive value in shell egg are small, the only report of any loss being for vitamin A which was found to decrease by 75% during storage for one year (Manville, 1926). The fall in nutritive value is not large, even when the egg is processed; there is some destruction of thiamine (30%) during spray-drying, but little or no destruction of vitamins A, D, riboflavin, nicotinic acid or pantothenic acid (Klose, Jones & Fevold, 1943; Cruickshank, Kodicek & Wang, 1945).

4. Milk

A large part of the liquid milk supply in Great Britain is pasteurized, i.e., held at 150° F. for 30 min. This mild treatment causes only a small decline in nutritive value (20% loss of ascorbic acid; 10% loss of thiamine) (Kon, 1944).

Milk is also dried, either by the spray or roller methods. The loss of vitamins during spray-drying is about the same as in pasteurization, though there is also a small decline (5%) in the biological value of the protein (Kon, 1944). The nutritive value of roller-dried milk is usually slightly inferior to that of spray-dried milk.

Milk products in which less water is removed fall into two categories: evaporated and sweetened condensed milks. Evaporated milk is prepared by removal of water in vacuo at low temperatures; but the concentrated milk is then sterilized in cans at 240° F., which causes large losses of ascorbic acid (60%) and thiamine (50%). Sweetened condensed milk is not exposed during processing to temperatures above 100° C. and, accordingly, the loss of nutritive value is small (15% of ascorbic acid and 5–10% of thiamine) (Kon, 1944).[2]

5. Storage of Processed Foods

Care in processing food is wasted unless equal care is taken to preserve its quality during storage. Chemical changes occur in processed foods that are generally undesirable; flavour, colour, and texture deteriorate and nutritive value is reduced. Chemical change in stored food is accelerated by (i) a rise in temperature; (ii) the presence of oxygen; and (iii) an increase in the concentration of reactants. Hence it is clear that to prolong the storage life one must store at as low a temperature as is economically possible, and under conditions in which oxygen is excluded. Even so, chemical change may occur between major constituents of the food, and this becomes especially marked where concentrated dried foods are involved.

It is impracticable to gas-pack all processed foods for storage, and, in practice, this is restricted to foods packed in an inert atmosphere in sealed containers; and it is usually confined to dried products in which, owing to the large exposed surface, oxidative change would otherwise be rapid. With dried milk, eggs and vegetables, loss of the more easily oxidizable nutrients is likely to be large unless this is done.

Storage in nitrogen or carbon dioxide reduces the rate of loss of ascorbic acid from dried cabbage to one-third of that in air, and prevents any loss of carotene from dried carrot (Tomkins, Mapson, Allen, Wager & Barker, 1944). The interesting observation that the rate of oxidation of carotene in dried carrot strips stored in air may be reduced by half if the strips are scalded in a dilute starch solution was first made by Tomkins, Mapson & Wager (1946). In their studies the moisture content of the vegetables had an important influence on the stability of ascorbic acid and carotene; the higher the moisture content the more rapid was the loss of these vitamins.

Storage in an inert atmosphere also prevents the development of rancidity in fatty foods: a change which affects the nutritive value, because the hydroperoxides formed by the

[2] See also Kon, *Brit. med. Bull.* 1947, **5**, 170.—Ed.

oxidation of unsaturated fatty acids destroy the fat-soluble vitamins (A, D and E) and the water-soluble ascorbic acid and biotin. It is therefore essential to gas-pack products such as dried milk or eggs, if deterioration in culinary quality and nutritive value is to be avoided (Cruickshank *et al.* 1945).

Even foods stored at low temperatures lose their nutritive value. The most prominent changes in animal, fish, and dairy products are due to rancidity and the consequent effect on nutritive value (Dubois, Tressler & Fenton, 1940). There is little change in vitamins of the B group during storage in the frozen state (Rice, Fried & Hess, 1946; Rice, Squires & Fried, 1948; Lee, Brooks, Pearson, Miller & Volz, 1950; Cook, Morgan & Smith, 1949; Morgan, Kidder, Hunner, Sharokh & Chesbro, 1949).

Jenkins and his co-workers (Jenkins, Tressler & Fitzgerald, 1938; Jenkins, Tressler, Moyer & McIntosh, 1940) studied the loss of ascorbic acid from frozen vegetables stored at temperatures between 16° F. and −40° F. At 16° F., ascorbic acid was totally lost within three to six months; losses were correspondingly reduced as the temperature of storage was lowered and, to retain the maximum amount of ascorbic acid for six to ten months, it was essential to store at −20° F.

To a lesser extent, carotene may be lost during cold storage. Zscheile *et al.* (1943) found that, at −4° F., the loss of carotene was small within 6 months, but increased considerably during a further 5 months, when losses as high as 36–49% were found. Thiamine and riboflavin are more stable: negligible losses have usually been reported after storage at −4° to −10° F. over periods of one year, although Farrell & Fellers (1942) encountered a 22% loss of thiamine from green beans after storage for 12 months at −4° F.

Reports on the stability of ascorbic acid in frozen fruits are conflicting. Isham & Fellers (1933) found no loss of ascorbic acid from cranberries or orange juice stored at 0° F. for 9–20 months; but Mayfield & Richardson (1943) reported loss as high as 50–70% in strawberries held at 0° to −10° F. for 4–6 months, and Todhunter & Robbins (1941) found a 23% loss of the vitamin in frozen raspberries stored at 0° F. for 9 months. A figure more nearly representative of losses in normal practice would appear to be about 15–20% for fruit frozen in syrup and stored for 6–12 months at 0° F. (Adam, 1949). There is little loss of ascorbic acid if the fruit is thawed shortly before it is eaten, but losses may be high if it is thawed and is then kept, even for a short time above 0° C.

Vitamins in canned foods are fairly stable unless the temperature of storage is excessively high. Cameron & Esty (1950) summarized work which showed that losses of ascorbic acid, carotene, thiamine, riboflavin, or nicotinic acid, from several vegetable foods, were 15% or less within two years at 50° F. We may conclude that canning offers an effective means of preserving these nutrients over an extended period of storage. The greater stability of vitamins in canned foods, compared with dried foods, is no doubt due to their lower concentration in the former, as well as to the elimination of oxygen which results from the canning procedure. The loss of ascorbic acid in canned fruit during storage is also small (Adam, 1949).

6. Newer Methods of Processing

A review of this nature is incomplete without some reference to newer methods of processing which, while at the moment of academic rather than commercial value, may be successfully developed in the future. We have seen that one of the prime losses of nutritive value during traditional methods of processing is in the scalding or blanching treatment by water or steam. It is therefore interesting that heat treatment by high-frequency radiation has been found to be successful in inactivating enzymes, and in reducing the loss of water-soluble nutrients (Moyer & Stotz, 1947; Proctor & Goldblith, 1948). There are other advantages: notably that rapid and uniform heating of large masses of material may be achieved. The authors emphasize that, unless the radiation treatment is adequate, very severe losses of ascorbic acid and carotene may occur during storage.

A further development is the proposal to use ionizing radiations, such as cathode rays, γ rays, and x rays, for preserving foods; this they do by killing micro-organisms. There is ample evidence that the treatments envisaged can cause extensive destruction of vitamins in dilute solution, mainly through attack by highly reactive free radicals formed from irradiated water molecules, and similar effects on fat-soluble vitamins in non-aqueous solvents have been observed. The effects in foods are less clear, however, since the destruction of vitamins and other nutrients is decreased considerably by the presence of other reactive organic constituents. Suitable adjustment of the processing conditions effects a further decrease, and it appears, as a result, that the final destruction of vitamins in many foods is small, particularly with treatments which do not aim at complete sterilization. In some cases, however, especially when the initial vitamin content is low, the effect of a sterilization treatment can be considerable (Kung, Gaden & King, 1953), and much further investigation is required. It should be added that, in this type of process, destruction of vitamins and other essential nutrients is not the only potential hazard to the health of the consumer. New compounds of an undesirable nature might be produced, as a result of radiochemical action, and the process will therefore need to be carefully examined before it can be adopted as a method of sterilizing food. The subject has recently been reviewed by Hannan (1955).

7. Conclusions

Some loss of nutritive value is inevitable, however carefully food is processed. How serious this is depends on the food itself, on its natural vitamin content and its importance as a source of a particular nutrient in the dietary. It is unimportant if ascorbic acid is lost from meat or eggs, since these foods are unimportant sources of this vitamin; on the other hand, a large loss from processed fruit or vegetable foods is more serious, for one of their chief nutritive properties is their content of the antiscorbutic principle.

The loss of nutritive value in processing has to be set against similar losses that occur when fresh food is cooked. Apart from the few foods that are eaten raw, the same factors which operate to reduce nutritive value during processing also operate during domestic cooking. Processed foods may also have to be cooked but, since they have been subjected to heating during processing, the cooking needed is less than that required for fresh foods. Hence the further loss of oxidizable and extractable nutrients is usually less than in fresh foods. A common error made with most processed foods is to cook them too long and, if this is done, unnecessary losses may occur (Jones, Wood, Phillips, Fenton & Harris, 1944). Table I, compiled from publications by several workers (Adam, 1949; Barker *et al.* 1947; Olliver, personal communication,

TABLE I. COMPARISON OF THE AMOUNT OF ASCORBIC ACID RETAINED IN COOKED PEAS PROCESSED IN DIFFERENT WAYS WITH THAT IN COOKED FRESH PEAS

The reduction of ascorbic acid at various stages in processing is also shown. The figures given are averages

Frozen peas		Canned peas		Air-dried peas		Freeze-dried peas		Fresh peas	
Stage of processing	Percentage retained	Stage of processing	Percentage retained	Stage of processing	Percentage retained	Stage of processing	Percentage retained	Stage of processing	Percentage retained
Scalding	75	Scalding	70	Scalding	75	Scalding	75	—	—
Freezing	75	Canning	63	Drying	45	Drying	70	—	—
Thawing	71	[Diffusion into liquor]	40	—	—	—	—	—	—
Cooking	**39**	**Heating**	**36**	**Cooking**	**25**	**Cooking**	**35**	**Cooking**	**44**

1951), compares the total loss of nutritive value in terms of one nutrient, ascorbic acid, from one typical vegetable food (i.e., peas), during processing and cooking with that during cooking only. The figures are averages, and losses in practice will be greater or less than those recorded, depending on the methods of processing or cooking employed. Nevertheless, they serve to show that, where care is exercised in processing, the antiscorbutic value of the food as eaten is not greatly inferior to that of the freshly cooked product. Although these figures relate only to ascorbic acid, the retention of other water-soluble nutrients should be at least as good, and of fat-soluble nutrients even better.

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VITAMINS IN NUTRITION: ORIENTATIONS AND PERSPECTIVES

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Having had the advantage of studying all the other contributions before writing this paper, I have been able to include a few of the thoughts prompted by the foregoing papers. I have perforce become something of an epilogist; in this role, by quoting from the remarks, writings and work of Sir Edward Mellanby, I have attempted to round off our appreciation of his achievements in the science of nutrition and to show our indebtedness for the opportunities and facilities for research many of us owe to him.

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He remarks on the comparatively recent introduction of "sallets" (salads).

Whilst life in English villages at the end of the eighteenth century was by no means all "beer and skittles" there was a degree of civilization—a measure of improvement of rural welfare such as is aimed at in underdeveloped areas in what are nowadays sometimes called community development projects. "Medicine", Sir Edward Mellanby used to say, "has replaced the Bible as the spearhead of penetration" in such projects, and applied nutrition work is often an important element if not sometimes the point of this spearhead. By contrast there is a squalid side to a form of civilization, for which I think the term sophistication is more appropriate; it is usually associated with urban areas and often features some form of malnutrition. In a statement in one of his earlier papers on rickets Mellanby (1919) drew attention to the fact that children with bony deformities represented only a small part of the cases affected. Examination of children in London County Council schools led to the conclusion that 80% of them had rickets. Furthermore, ". . . in poor urban districts . . . where rickets is rife the death-rate in children varies from 100 to 300 per 1,000".

In his next paper on rickets Mellanby (1920) quotes the results of a survey of schoolchildren in Leeds in 1902 by Dr William Hall who found that half the Gentile children from a poor district had rickets, and 60% had bad or backward teeth, whilst only 7% of poor Jewish children had rickets and

25% had bad or backward teeth. Hall concluded that the inferior physique of the Gentile children was of purely dietetic origin. The evidence presented leaves no doubt that the Jewish children not only had more vitamin D in their diets than the Gentile children but that their diet was richer in many other nutrients.

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Admittedly many striking improvements in the health of many countries have been effected in the past 25 years through advances in the knowledge of the science of nutrition; so much so that digging up the past may seem out of place in a symposium devoted to recent work in the subject. There are, however, communities still to be found in the world in which half the children born die in early life (Platt, 1953a). Malnutrition undoubtedly contributes to such dreadfully high mortality rates.

2. Some Nutritional Notions

a. Relation of Food and Health, and of Vitamins and Deficiency Disease

Sir Robert McCarrison, one of the British pioneers in nutrition, has claimed for McCay (1912) the credit of being the first to obtain proof, in his study of Indian races, of a relation between food and health. McCarrison (1953) holds the view that, before this work, statements on the role of food as "the paramount influence in determining man's general physical endowment, powers of endurance and of resistance to disease" were only expressions of opinion. Nevertheless, in the history of the study of nutritional disorders, there are numerous examples, many of them well known, of the association between disease and faulty nutrition. For centuries the Chinese have recognized the connexion between diet and disease. Hu Se-Huei who was Imperial Dietician from 1314 to 1330 wrote a book on the principles of correct diet, the second volume of which is devoted to the cure of diseases by diet (see Lu, 1939b).

Mellanby (1944) has reminded us that Chatin in 1850 related the development of simple goitre to a deficient intake of iodine. The concept that disease could be due to the shortage of something took time to emerge and to spread in face of the notion that some agent—poison or parasite—was necessary for the causation of disease.

Interesting historical accounts[2] of the growth of knowledge of several nutritional diseases are to be found in a Report of the Accessory Food Factors Committee (Medical Research Council, 1932; see also Chick & Hume, 1956). In this Report Grijns is given ". . . the credit of first picturing clearly [in 1904] what is to be justly called a deficiency disease, as well as that of first investigating the properties and distribution of a vitamin . . ."; Funk, who in 1912 introduced the word "vitamine", also ". . . by giving objectivity to the conception of 'deficiency', in the right sense . . . helped to a public and professional recognition of the reality of deficiency disease; a recognition which at that time came none too readily."

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Whilst there is no doubt that "where and in proportion as you can use experiment, science advances rapidly", there has perhaps been insufficient recognition of the stimulus derived by research workers in nutrition to the related clinical problems and to the extent to which there has been interplay between laboratory and field work. There are numerous examples of the stimulus to laboratory work arising out of practical problems and, in return, benefits to mankind accruing from experiments in the laboratory. The point is made here in the hope that encouragement and facilities will continue to develop for exchanges of opportunity and experience between field and laboratory workers, especially where there are many problems and few or no laboratory facilities.

c. Zymotic Agents in Nutritional Disease

The introduction into medicine both of the germ theory of disease and of the concept of nutritional deficiency disease was encumbered with controversy. The latter was also complicated by the temptation to attribute the causation and pathology of dietary deficiency diseases to zymotic factors in the patient or his food. When I began research on beriberi in a modern hospital in Shanghai in 1933, I found it was still the practice to sterilize the bedding of all patients who died from the disease—on the assumption that it was infectious. Years of work and much effort must have been expended in the Far East on investigations into the supposedly zymotic nature of the causal agent.

One by-product of the germ theory has been the drive for "clean" food; this I maintain should not involve the destruction of organisms that may be beneficial to the consumer. In the course of some kinds of processing of foods the nutritional value may indeed be enhanced, as in the germination of grains and pulses or in fermentation (Platt, 1954c); such enhancement I have called "biological ennoblement" (Platt, 1946a; Platt & Webb, 1946). It would seem to be wrong if, as in the case of Kaffir beer production, a process is condemned as "unclean", and suppressed without at least making sure that the product is replaced in the diet by something equally good (Platt, 1955c).

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occur without affecting the utilization of many of the B vitamins. I have observed the need for an increase in the dose of vitamin B_1 in the treatment of beriberi when the disease is complicated by fever (Platt, 1938), and I have reported (Platt & Gin, 1934) that half of a series of patients with subacute beriberi first noticed their symptoms after a non-specific infection.

The indebtedness of the ruminant to its microflora is well known; to a much lesser extent, man and those animals with similar types of alimentary economies are supplied with some accessory food factors from intestinal microbial syntheses. The magnitude of synthesis in the human alimentary tract may be gauged from the fact that about 8 g. of bacterial matter are produced daily (Platt & Webb, 1948); the possible side-effects of this synthesis on health and disease are virtually unexplored. More will be heard on this subject in the future no doubt, since pigs treated with antibiotics, and indeed animals in the same piggeries as those treated, can utilize food much more efficiently than untreated animals (Catron, 1955). So striking is this effect that feeds containing antibiotics are being widely used in commercial pig farming.

d. Social and Economic Factors and Nutrition Education

Whilst ignorance and misconception may have accounted for some of the tardiness in the advancement and application of nutritional knowledge, poverty is often associated with ignorance in contributing to malnutrition. Wealth is not merely a matter of money, and a shift from a subsistence to a money economy, especially where ignorance remains, has often been followed by outbreaks of frank deficiency disease.

Nevertheless "industrialization"—including such developments as plantations of "cash" crops—have sometimes had beneficial effects on the health of the workman. Industrial enterprises in various parts of Africa afford welcome examples of the efforts of employers; in this field Orenstein in the Union of South Africa was a pioneer. The benefits of good feeding of the labourers in the mines of Johannesburg are still being demonstrated (Walker, Fletcher, Reynolds, Bersohn & Sonnenfeld, 1956).

It appears as if the control of beriberi in Malaya might be another example (see Institute for Medical Research, Federation of Malaya, 1951a). As cited in the section on beriberi in this Report, Fraser and Stanton had in 1911 reached the conclusion that ". . . rice is rendered harmful by the milling and polishing process to which it is subjected in the preparation of white polished rice. In this process there is removed from the grain some substance of high physiological importance in metabolism . . ." As quoted in this Report, in 1923 Stanton wrote: "The fall in the incidence of beri-beri in the Federated Malay States during the past ten years has been so notable that simple measures will suffice within a few years to abolish the disease". In so far as the recorded admission of patients with beriberi into government hospitals tells the tale (see fig. 1), a reduction had begun about 1905—this was twenty years before Jansen and Donath isolated vitamin B_1. Good business men no doubt saw the point of the advice of the principal Medical Officer (Institute for Medical Research, Federation of Malaya, 1951b):

> . . . a labourer needs a definite supply of [nutrients] to enable him to work . . . from the human point of view but also from the business aspect it is necessary that this supply is adequate. Expenditure on prevention of illness is soon repaid with large interest . . .

FIG. 1. ADMISSION OF PATIENTS WITH BERIBERI TO GOVERNMENT HOSPITALS IN THE FEDERATED MALAY STATES 1895–1949

Based on fig. 40 of Studies from the Institute for Medical Research, Federation of Malaya (1951a)

Abscissae: calendar year
Ordinates: number of cases per 1,000 population

The fact that the attitude of a people towards food and disease and towards crop production and distribution should be taken into careful consideration when planning for improvement in health or agriculture has been recognized for only a very few years. Two studies of A. I. Richards (1932, 1938) were pioneering pieces of work. Read (1955) has recently published a number of papers based on her experience as a social anthropologist; and a volume edited by Mead (1953) is intended as a guide to those concerned with "purposive technological change". The latter work, suitably garbed, is now on sale in the drug stores throughout the USA.

At the third meeting of the Joint FAO/WHO Expert Committee on Nutrition (1953a), we expressed the opinion

> . . . that there is no better way of initiating a programme of community development (which includes greater food production) than by making a survey, the object of which is to determine the nature and magnitude of the problems to be solved, and also to discover what the villagers themselves know, and to establish good working relations between them and those who are seeking to help.

The closely related question of education in nutrition is too big a subject to tackle here. At the last meeting of the Joint FAO/WHO Expert Committee on Nutrition (1955a) it was felt necessary to reiterate the importance of training in nutrition in the medical curricula of various countries. In my own department some newcomers, overseas postgraduate students, were heard recently to say, "Why should we need to know about nutrition—we are clinicians"!

There is often a tendency to stress, in education work, the techniques of presentation and to neglect the content of the teaching. This is of course deplorable; there is no lack of fundamental knowledge; there is, however, often evidence of failure to adapt this knowledge to local conditions, foods and

customs. Papers on "Food production and extension methods" (Joint FAO/WHO Expert Committee on Nutrition, 1953b) and "Methods and content of education in nutrition" (Joint FAO/WHO Expert Committee on Nutrition, 1955b) will be of value to those interested in this subject.

3. Applied Nutrition

a. Vitamins in Therapeutics

Research workers are generally greatly interested in seeing the results of their work applied; this is clearly evident in the writings of contributors to this number of the Bulletin. There is, however, generally little delay in the application of vitamins to the treatment of disease. Sometimes the results of treatment are most dramatic. I lost every patient with acute fulminating beriberi admitted to my wards in a hospital in Shanghai in the three years 1933–35. During a visit to Batavia at the end of 1935 I obtained from Dr A. G. van Veen a small quantity of crystalline natural vitamin B_1; it was rarely necessary to give more than a single intravenous injection of only 5 mg. of this material to change the condition of the patient so markedly that he would be sitting up in bed a few hours later, smiling. Seventeen of the 21 patients admitted in 1936—of the type which formerly invariably died—recovered, the others were too near to death for the vitamin to have time to take effect (Platt, 1938); the raised blood pyruvate values took 10–15 hours to return to normal.

b. Preventive Measures

It is much more difficult to introduce simple, permanent and acceptable preventive measures to a community than to treat the sick. In parboiled rice 80% of the original vitamin B_1 and a substantial proportion of some other B vitamins are retained, whereas at least 80% of vitamin B_1 is lost in milling to a high bran yield. In my research Unit the methods of parboiling have been examined (Done, 1949), and a product almost indistinguishable gastronomically and in appearance from highly milled rice can be produced; but the difficulties of introducing parboiled rice in some communities where vitamin B_1 deficiency still occurs have not yet been surmounted. Similar difficulties have been encountered in introducing enriched rice (Food and Agriculture Organization, 1954b). On the other hand, it was not many years after Mellanby's discoveries concerning the causation of rickets that, when it became necessary to assay the potency of the first supply of calciferol for the treatment of human rickets, no child could be found in a search of the London hospitals who had not already been given effective treatment with cod-liver oil—some cases were eventually found in an area of unemployment in the industrial north.

One of the characteristically pithy dicta of Lord Boyd Orr, the first Director General of the United Nations Food and Agriculture Organization (FAO), is "We must have more of the right kinds of food". This is generally the key to the problem of applied nutrition and it was provided for in the nutrition work of the Health Organisation of the League of Nations in the "marriage of health and agriculture". The courting period before this marriage must have been a matter of centuries—as in, for example, the England of Gilbert White. The first step in setting up the United Nations Organizations was deliberately—and significantly—the Conference on Food and Agriculture at Hot Springs, Virginia, USA, in 1943. FAO, which grew out of this conference, has recently celebrated the tenth anniversary of its inauguration. Health as well as food and agriculture needs to be taken care of in making plans for world nutrition (Platt, 1944); co-ordination of nutrition work of FAO and the World Health Organization (WHO) is fostered through the work of the Joint FAO/WHO Expert Committee on Nutrition. Lord Horder was Chairman of the first meeting of this Joint Committee; he had a lively interest in nutritional matters and he had consented to contribute to this number of the Bulletin. Sadly his recent death prevented this. A recent meeting of the Nutrition Society (Nutrition Society, 1956) was devoted to the work of FAO and WHO, and some contributions of British nutritionists to these international activities have been the subject of a recent editorial (*British Medical Journal*, 1955).

At the national level there have been many achievements, some of them outstanding. Perhaps the most impressive was the success of the policy in the United Kingdom during the Second World War, planned by the War Cabinet Scientific Food Policy Committee and admirably administered by the Ministry of Food whose Scientific Adviser was the late Sir Jack Drummond.[3] Before this and arising directly out of activities of the Health Organisation of the League of Nations, was the work of the Committee on Nutrition of the Colonial Empire (see Platt, 1944). Their survey revealed problems throughout the Colonial Empire and led to a report (Committee on Nutrition in the Colonial Empire, 1939) which is still widely used and was "discovered" and turned to good account by the United Nations Interim Commission on Food and Agriculture (1945). Progress in nutrition investigations in Colonial territories was reviewed at the British Empire Scientific Conference (Platt, 1948), and researches since that time are noted in the reports of the Colonial Medical Research Committee (1947). The plan to assist Colonial territories to develop their own nutritional organizations is unfolding, and the efforts made from the territories' own staff are being supplemented by help in various ways from home-based institutions.

Tropical Africa has many nutrition problems; these have been discussed at two Inter-African Nutrition Conferences (Conférence interafricaine sur l'alimentation et la nutrition, 1949; Colonial Office, 1954) held under the auspices of the Commission for Technical Co-operation in Africa South of the Sahara (C.C.T.A.). The second Inter-African Nutrition Conference was held in conjunction with the third meeting of the Joint FAO/WHO Expert Committee on Nutrition (1953a), in the Gambia, British West Africa, in 1952, on the subject of malnutrition in mothers, infants and children. Nutrition research and related development work is now being co-ordinated through a group of "nutrition correspondents" for the Belgian, French, Portuguese and South African territories, and British territories, including the Federation of Rhodesia and Nyasaland.

c. Vitamin Requirements and Vitamin Content of Foods

Times of excitement over the discovery of new vitamins are almost if not completely past, and much of the effort in nutrition research has in recent years been spent on unexciting though necessary investigations such as the determination of the amounts of the various nutrients needed for health and in ascertaining how well these are satisfied from the food eaten. There is general agreement about the magnitude of the

[3] An account of the work of the Ministry of Food is given by Pyke (1944).

requirements for many vitamins, the principal differences between various "standards" being the objective, e.g. whether to satisfy the minimal needs of the individual or to provide amounts adequate for maintaining a good nutritional state in various representative groups of the population (British Medical Association, 1950) or to aim at "recommended dietary allowances", i.e. "not requirements, since [these allowances] represent, not merely minimal needs of average persons, but nutrient levels selected to cover individual variations in a substantial majority of the population" (National Research Council, 1953).

Assays of vitamins in food-stuffs have provided material for the compilation of tables which can be employed in the evaluation of dietary data obtained in various types of surveys.[4]

d. Nutrition Surveys

The objects of a nutrition survey tended at one time to be limited to a collection of data on food consumption or to an attempt at an appraisal of the state of nutrition of a community; or perhaps, by doing both, to try to establish a relationship between health—or disease—and the food eaten. Whilst this type of investigation may still be useful, I would generally subscribe to the view expressed in the quotation given above (see section 2d).

Much useful information can be obtained by the preparation of food balance sheets for a country. Data on food supplies are collected and examined in relation to the consumer's needs. On scrutinizing food balance sheets, discrepancies between supplies and requirements may be revealed, and we know enough now about practical nutrition to be able to prescribe proper measures to overcome them. It is still necessary, however, to supplement the work of the statistician and economist with at least two inquiries: (i) a study of methods of storage, of preserving, including cooking, and of distribution of food; and (ii) clinical assessment of an adequate sample of the population in order to determine the state of nutrition of the consumer.[5]

These inquiries require a sound training in nutrition: for the first, knowledge is needed of the distribution of vitamins in foods and such other properties as their stability during treatments, storage and preservation; for the second, it is necessary to know the effects on the body of insufficiency. Much has been written in recent years about methods of assessment of the state of nutrition, especially with respect to individual vitamins (see, for example, Zilva, 1944; Harris, 1956), and the Joint FAO/WHO Expert Committee on Nutrition at its second meeting (1951) prepared a guide on the assessment of the nutritional status for nutrition workers.

e. Food Technology

Research in modern food technology has been concerned particularly with the effects of processing on vitamin content, as stated in another contribution to this number (Mapson, 1956; see also Chick & Hume, 1956). Although the discoveries in this field apply in general to the treatment of foods in all parts of the world, comparatively little attention has been given to domestic procedures indigenous to many countries. In my experience, many of these are nutritionally sound and those of some communities, e.g. the Chinese, might be more applicable to such underdeveloped communities as Africa than are some techniques of modern food technology such as canning and refrigeration. Two examples of homely methods are the pickling of Chinese cabbage with rice bran and salt (some of the B vitamins from the bran are adsorbed on to the leaves) and the manufacture on a village scale of soya-bean curd which is good to eat and, so far as is known, is non-toxic (as contrasted with soya-bean meals, which have generally to be "sold on the back" of some other food-stuff—or so-called soya "milks" which may contain some toxic substances which appear to be absent from the curd).[6] There are numerous other examples in the various types of fermentation (Platt, 1954c).

The retention of the vitamins and other nutrients in the staple foods of those who rely on the staple as the mainstay of their diet is of the greatest importance; quite small losses from the staple may appreciably and sometimes seriously affect the adequacy of the diet in some nutrients. Reference has already been made to the importance of the retention of vitamin B_1 in rice and, during the Second World War, much research went into securing a national loaf of high nutritional value (Chick & Hume, 1956). People with maize as a staple article of diet may have pellagra, though I have worked throughout the year amongst Africans who have a high proportion of their calories, sometimes as much as 80% of their dietary total, from maize, and yet no cases of frank pellagra occurred. The aetiology of this disease is acknowledged to be complex (Joint FAO/WHO Expert Committee on Nutrition, 1955a) and is not completely understood. As reported in another contribution (Coates, Kon & Porter, 1956), there is evidence that alkali treatment of maize liberates bound nicotinic acid; lime treatment of maize, as used for the making of flour for tortillas, might be a desirable procedure to extend to other maize eaters. However, we have found (Pellett & Platt, unpublished) that lime treatment destroys riboflavin and thiamine. In African dietaries the riboflavin is often barely enough to prevent the occurrence of signs of deficiency, and it would clearly be undesirable to risk reducing the amount of this substance still further in maize diets, unless alternative sources of supplies could be included.

f. Fortification

Since the manufacture of vitamins on a large scale by chemical synthesis, supplies of many are cheap and abundant. Consequently, there has been for some years now pressure from several interested commercial enterprises for the inclusion of synthetic nutrients in food products. As the synthetic substances are identical with the products isolated from natural sources and have the same effects in the body, the artificial substance can be substituted without legitimate object-

[4] See, for example, *Nutritive values of wartime foods* (Medical Research Council, 1945); *Tables of representative values of foods commonly used in tropical countries* (Platt, 1945b); *Food composition tables for international use* (Food and Agriculture Organization, 1949); and *Food composition tables—minerals and vitamins, for international use* (Food and Agriculture Organization, 1954a).

[5] A limited number of copies of some typical surveys and a memorandum on the method of making a dietary survey are available at the Applied Nutrition Unit, London School of Hygiene & Tropical Medicine, Keppel Street, London; also a few memoranda, prepared for Nutrition Officers and others in British Colonial Territories, on problems such as are commonly found in tropical countries, are available. Brief accounts of the work of this Unit and a list of publications are to be found in the Annual Reports of the London School of Hygiene & Tropical Medicine. Those interested in comprehensive all-the-year-round nutrition surveys will find of value and interest two reports by Mrs G. M. Culwick (1950, 1951), who was for many years associated with our work. A film strip, "Improving the Food Supply", based on some of our field work, is included in the British Council Study Box—"Health in Hot Countries". This box contains study material relating to many aspects of hygiene and tropical medicine, and is available on free loan on application to the British Council, 65 Davies Street, London, W.1, or to any British Council office.

[6] Soya-bean is used traditionally for the feeding of infants in China (Platt & Gin, 1938) and is recommended for mixed feeding of infants (Platt, 1954b).

ions. The problem of the fortification of foods with vitamins is, however, generally more complicated than that of simple substitution.

There is a large measure of agreement between nutritional scientists[7] that it is desirable to meet nutritional needs as far as is practicable from natural foods, and in Britain indiscriminate fortification, with vitamins or other nutrients, is considered unnecessary and unsound apart from a few instances in which the practice is permitted. It is not unreasonable to apply our present-day knowledge to restoring the nutritive values to food-stuffs that have been highly purified, e.g. iodine to pure preparations of sodium chloride (there is of course ample iodine in the cruder sea salt) or to make margarine as nearly as possible nutritionally equivalent to butter by the addition of vitamins A and D. It is another matter to refine wheat flour to such a degree that the diets of some—especially the poorer members of a community who may eat large amounts of bread—have barely enough of some of the vitamins contained in the original wheat berry, and it is not justifiable to argue that the addition, as synthetic substances, of some of the nutrients removed necessarily restores the value of the food. If it is argued that refined white flour has good nutritive value, then there is no case for enrichment; if there is alleged to be a case for enrichment, then it is better to mill the grain to yield a good flour and retain as much as possible of the natural complex of nutrients as is consistent with good baking qualities and acceptability to the consumer.

Intelligence and common sense are based on the exercise of a sense of proportion; this has sometimes been lost or distorted in the employment of vitamins. Over-enthusiasm, high-pressure advertising and indiscriminate prescribing have led to abuse of these substances, sometimes with detrimental effects on the consumer, and generally to bring discredit on the subject and even make it appear ridiculous. A good sense of proportion is still needed in the application of the knowledge of vitamins and indeed in the whole field of applied nutrition, especially since it so readily can become a field for faddists. Scientists and members of the medical profession can give a lead to the public in these matters, and they should help to determine government policy. As Sir Edward Mellanby writes in the earlier number of this Bulletin on nutrition (Mellanby, 1944):

> . . . no nutritional policy adopted by governments can be wrong if it places the health and the needs of the community as its first and guiding principle.

4. Biological Problems

a. The Function of Vitamins

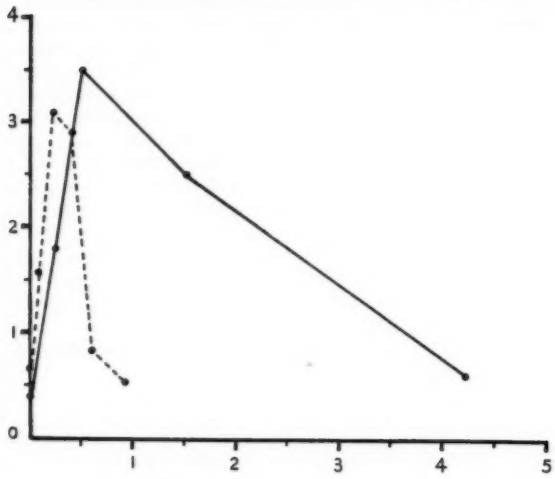
Sir Edward Mellanby, writing in 1944 about the biological side of vitamin research, asks ". . . can it be said why these substances are essential and/or in what way they work?" He continues ". . . there is hardly any knowledge of how the vitamins work in performing their functions"; and, having discussed the ways in which vitamin D may work, he states that ". . . there will still remain the problem of how it performs these functions." The biological problem was one of the greatest interest to him and a constant challenge which he was contesting, to some purpose, to the day of his death. The subject is, of course, enormous, and throughout this number contributors have not hesitated to speculate on the mechanism of the action of the various accessory food factors. Two illustrations may be given of the status of these problems.

Beriberi and the toxicity of pyruvate. Peters (1953) has given an account in this Bulletin of his discovery of the need in tissues for vitamin B_1 for the metabolism of pyruvate. Twenty years ago, in the same year and independently of two of Peters' associates (Thompson & Johnson, 1935) who found evidence of the accumulation of pyruvate in the blood of avitaminotic rats and pigeons, Platt & Lu (1935), inspired by the results of earlier work of Peters, found pyruvate in the blood of Chinese patients with fulminating beriberi; and pyruvic acid was isolated as the 2 : 4-dinitrophenylhydrazone, and quantitatively analysed. Johnson (1936) succeeded in isolating enough hydrazone from pigeons' blood for analysis in the following year. Platt (1938) gave an account of observations on the symptomatology of beriberi, including the effects of such factors as increased metabolism due to ingestion of carbohydrates, fever and work; and the role of stress factors in localizing the lesions in beriberi and other deficiency diseases is discussed in earlier numbers of this Bulletin (Platt, 1944, 1945a). Evidence of the occurrence of pyruvic aldehyde (methyl glyoxal) was reported in our first paper, and our observations (Platt & Lu, 1936) ". . . led us to suspect that there is a more profound toxaemia in the presence of appreciable quantities of methyl glyoxal" (cf. Salem, 1954, quoted by Sinclair, 1956, and see Platt & Lu, 1936, figs. 1, 3). Further details of the levels found in patients with beriberi are given by Platt & Lu (1939) and of the effects of exercise on blood pyruvate by Lu & Platt (1939). The results of experiments on animals—including work on the behaviour of injected pyruvate—are in papers by Lu (1939a) and Lu & Needham (1939).

On many of our observations made in this early work, the statements by Sinclair (1953, 1956) are in agreement; where there is disagreement, the issue is still in my view debatable. In view of recent work (Draper & Johnson, 1951), my statement (Platt, 1951) that the manifestations of beriberi are not due to effects of pyruvate on the organism may have to be modified. The literature is too large to examine fully here; but certain points may be made briefly. A man may die with a blood level of 3 mg. pyruvic acid/100 g. if he is not given 5–10 mg. of thiamine. Although it is likely that the concentration of pyruvate is higher in some tissues than in the blood, assuming uniform distribution throughout the body, this level would be of the order of 0.03 g./kg., whereas Dobrovolskaïa-Zavadskaïa (1946) gave 0.1 g. pyruvic acid to a mouse weighing 12 g., causing "le béribéri expérimental" and causing death in 17 hours; this dose is at the rate of over 8 g./kg. body-weight. It might be expected that the amount of pyruvic acid required to produce toxic symptoms or death would be much less in animals with vitamin B_1 deficiency, but the reported data on this point are not consistent. It is, however, quite clear to me that the behaviour of endogenously produced pyruvate is different in the normal subject from that in the subject deficient in vitamin B_1; this is illustrated in fig. 2, in which are shown the blood levels at intervals after exercise of a normal person (Johnson & Edwards, 1937) and of a patient with beriberi in whom the blood pyruvate reaches similar levels. The return to normal values is much slower in the patient with beriberi than in the normal subject.

[7] Professor L. A. Maynard, until recently Chairman of the Food Nutrition Board of the National Research Council, USA, has discussed (Maynard, 1955) the attitude of official bodies in the USA to the addition of specific nutrients to foods, and in another paper (Maynard, 1954) he has examined the case for "glamourizing" milk by "multifortification".

FIG. 2 ACCUMULATION AFTER EXERCISE, AND RETURN TO NORMAL LEVELS, OF PYRUVATE IN BLOOD, IN HEALTHY SUBJECT AND IN PATIENT RECOVERING FROM BERIBERI



Abscissae: hours after exercise
Ordinates: pyruvic acid (mg./100 g. blood)

•••••••• Data for healthy subject, from Johnson & Edwards (1937)

———• Data for patient with beriberi, from Lu & Platt (1939), case no. 3198

Whatever may be the contribution to the syndrome of vitamin B_1 deficiency of pyruvate or of other carbonyl compounds—e.g., methyl glyoxal—there is abundant evidence that the function of cells deficient in vitamin B_1 is impaired, and it may be that, as stated by Peters (1953), "... the failure in energy supply at the pyruvate step must distort the metabolism of the brain cells and throw the nervous system out of gear". It is of some interest to report that I have some data (unpublished) showing that patients with fulminating beriberi, who fail to respond to therapy with vitamin B_1, may have a low—sometimes a very low—blood sugar; they may have convulsions shortly before death, which at the time I thought to be like those of hypoglycaemia. Notwithstanding the knowledge we possess of the intimate mechanism of the biochemistry of the function of vitamin B_1, which has progressed further than that of all the other vitamins, there still remains a biological problem.

Ascorbic acid and the synthesis of mucopolysaccharide. Here I wish to draw attention briefly to some work in my research Unit on the intimate function of vitamin C, which is worthy of consideration in conjunction with the statements made by several contributors on this subject (see also Wolbach, 1953). We were responsible for examining the experimental wounds made in the Sheffield volunteers (see Chick & Hume, 1956) in the ascorbic acid deficiency experiment. Some experiments were made on guinea-pigs, in the course of which Penney & Balfour (1949) traced the failure of wounds to heal in scorbutic animals to the inability of the animal to synthesize mucopolysaccharides in the wound. Within 12 hours after injection of ascorbic acid to depleted animals mucopolysaccharides appear.[8] This is then followed by the normal sequence of events in wound healing, including the development of normal fibroblasts, the production of reticulin and the growth of capillaries.

Vitamin imbalance and delayed effects on the young. Richards (1945) has reported the results of some feeding experiments with female rats in which she found that the addition of vitamin B_1 to a poor diet consisting mainly of white bread produced fits in the offspring; the fits resembled those seen in pyridoxine deficiency and were prevented by giving pyridoxine. These observations are, I think, of considerable importance and they have not been given the attention they deserve. Richards gives other examples of B vitamin imbalances which have been reported.

This work I link in my mind with that of Hartroft & Best (1949), in which it was found that acute choline deficiency in the young rat may result in hypertension, but only in later life and after a period equivalent in man to about 20 years. I have discussed elsewhere (Platt, 1955a) the evidence for the possibility that disorders in early life may produce tissue changes at an advanced age; it is a subject for much more research.

b. Vitamin, Protein and Amino-Acid Interrelationships

For many years I have given attention to the problem as to the extent to which some manifestations of dietary deficiency diseases are due to vitamin insufficiency on the one hand, or to shortage in the diet of proteins (or of individual essential amino acids) on the other (Platt, 1946a, 1946b). In an article on the cutaneous manifestations of dietary deficiencies (Platt, 1945a), the vitamins were mainly considered; in a recent discussion on the skin in malnutrition (Platt, 1953b), due consideration was given to other components of food, especially proteins. Several contributors to this Bulletin have included so much material on relationships between vitamins and amino acids that I abandoned my original assignment which was to contribute a paper wholly on the subject.

One interesting example not mentioned is the demonstration by Ludovici, Axelrod & Carter (1951) of a sparing effect of pantothenic acid on methionine in the synthesis of immune bodies. The observation of Sarrett & Perlzweig (1943), which has been confirmed, that the ability of the liver to retain riboflavin is determined by the amount of protein in the diet (or in the liver) is important in the understanding of the need for balance in the diet and the tissues.

Protein malnutrition is a subject of great interest at the present time; in a lecture on this subject (Platt, 1956b) I have commented on the work of Frontali (1954), who has nicely "dissected off" from the syndrome of protein malnutrition the clinical evidences of deficiencies of vitamins A, B_1, nicotinic acid and pyridoxine. I believe rickets in Great Britain was sometimes a complex of at least vitamin D deficiency and protein malnutrition. I recall treating a small bloated child with vitamin B_1 in China in 1936 and found, after the enormous oedema subsided, that the child was grossly marasmic. There is little doubt, as might be expected if protein malnutrition is produced in the young when it eats the same diet as the adult, that the clinical picture will be, so to speak, coloured with signs of the prevailing local vitamin deficiency. No doubt the vitamin deficiency will be produced

[8] Coloured photomicrographs illustrating some of these observations on the effect of vitamin C on wound healing are reproduced in the book on cytology by Pearse (1953).

in protein malnutrition only when the vitamin is low relative to the amount of protein, except when other factors, such as a relatively high level of carbohydrate in the diet, increase the requirement for a particular vitamin. Conversely, when rehabilitating a patient suffering from protein malnutrition, it is essential to provide along with the protein an appropriately balanced supply of other nutrients. It is interesting to note here that Piéraerts (1950) failed to evoke a response to folic acid until enough protein had been given to a patient to clear his oedema.

5. The Concept of Balance

Attention has been drawn by Sir Edward Mellanby (1944) to the fact that Pasteur's remarkable work not only had a suppressive effect on the development of the notion of dietary deficiency as a factor in disease (see section 2c), but that it also had "a similar influence on the attitude to Claude Bernard's hypothesis that many diseases are due to the abnormal working of bodily processes which, when physiological or optimal, result in good health". With the name of Claude Bernard is immediately associated the concept of the constancy of the internal environment in the body. One of the striking features in many of the papers in this number of the Bulletin is to my mind the beauty of the picture that is emerging of the balance of reactions and metabolites (see also, for example, Krebs, 1953). We are indebted to Sir Rudolph Peters for the concept of the biochemical lesion which has contributed so much to the understanding of disturbances of metabolic equilibria in malnutrition. Peters' (1955) recent development of the idea of the unity of constellations of enzymes will be an important concept for research workers in the future.

The importance of balance of nutrients in the dietary can scarcely be overstressed; it is sometimes overlooked, though the term "balanced diet" is a familiar one. Its effect is of course reflected in the balance of metabolism in the consumer.

The need for balance should be recognized in applied nutrition—in a balance of the economy of a community, of crop and livestock production and in social and educational matters (Platt, 1953a). Possibly the most important balance of all to secure is equilibrium between the size of the world population and the world's resources of food and other commodities (Platt, 1954a; Political and Economic Planning, 1955).

* * *

Hippocrates, writing about such matters as "the difference to a man's body whether bread be of bolted or unbolted flour" says:

> But how could he who has not considered these truths, or who considers them without learning, know anything about human ailments? For each of these differences produces in a human being an effect and a change of one sort or another, and upon these differences is based all the dieting of a man, whether he be in health, recovering from an illness, or suffering from one. Accordingly there could surely be nothing more useful or more necessary to know than these things, and how the first discoverers, pursuing their inquiries excellently and with suitable application of reason to the nature of man, made their discoveries, and thought the art worthy to be ascribed to a god, as in fact is the usual belief.

The modern version of this might be the definition of the main function of the Medical Research Council to be found in the last report (Medical Research Council, 1949) issued whilst Sir Edward Mellanby was its Secretary: the promotion of "scientific investigations for the acquisition of knowledge likely to be of value for the prevention, diagnosis, and treatment of disease and for the maintenance of normal health and full human efficiency".

It may not be inappropriate to conclude this number of the Bulletin by relating an incident that occurred in an interlude during a meeting of the War Cabinet Scientific Food Policy Committee. A junior member of the group was complaining about Mellanby's handling of a situation—probably pushing through some important measure, regardless of the feelings of the aggrieved person—when an eminent member of the committee quietly interjected, "Mellanby—all I can say is, it is a pity there aren't more like him".

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International Congresses

The 4ÈME CONGRÈS INTERNATIONAL DE NUTRITION will be held in Paris, from 24 to 29 July 1957, under the aegis of the Union Internationale des Sciences de la Nutrition, and the chairmanship of Professor Terroine. Normal and pathological aspects of nutrition of both man and animals will be considered. The Congress will include sections on: nutrition and lactation; nutrition and reproduction; nutrition and skin; nutrition and digestive flora; nutrition and old age. Further information may be obtained from: 4ème Congrès International de Nutrition, C.N.E.R.N.A., 71 Boulevard Péreire, Paris 17e, France.

The SECOND INTERNATIONAL CONGRESS OF DIETETICS will be held in Rome, from 10 to 14 September 1956. The Italian Dietetic Association will be the host of the Congress. The programme is in four main sections, and will cover: recent findings of nutritional research, methods of education in nutrition, principles and problems of feeding large numbers of people, and problems of training dieticians. The address of the Secretariat and Congress is: Associazione Dietetica Italiana, via dei Penitenzieri 13, Rome, Italy.

Notes on Contributors

PROFESSOR ADRIEN ALBERT is Head of the Department of Medical Chemistry in the Australian University, Canberra. While awaiting the completion of the Postgraduate Medical School there, he and his colleagues have established the Department temporarily in London. Prior to taking up this appointment in 1949, Dr Albert was on the staff of the Wellcome Research Institution, London, and before that, from 1938 to 1947, he taught in Sydney University. During the Second World War, he was Adviser on Medical Chemistry to the Medical Directorate of the Australian Army, and was one of a group who maintained the supply of drugs for the armed services, usually by new syntheses with raw materials available in Australia. Dr Albert graduated in Sydney in 1932, and later obtained the London D.Sc. His present work is concerned with improving knowledge of the chemistry of pteridines and purines, correlating physical and chemical properties in heterocyclic substances, quantitatively investigating the metal-binding properties of chelating substances, and generally attempting the correlation of physicochemical properties with biological action. Apart from many original research papers dealing with these topics, two books by Professor Albert have appeared: *The acridines* (1950) and *Selective toxicity* (1950).

PROFESSOR C. H. BEST is Director of the Banting and Best Department of Medical Research, and Head of the Department of Physiology, University of Toronto. He is also Honorary Consultant to the Connaught Medical Research Laboratories, University of Toronto, a member of the Research Defence Board of Canada, and of the Scientific Advisory Committee of the Nutrition Foundation. He is the first President of the International Union of Physiological Sciences. In 1921 Professor Best and the late Sir Frederick Banting were the co-discoverers of insulin. He was also the discoverer, with various colleagues, of the enzyme histaminase and of the vitamin choline, and he initiated the work which led to the purification of heparin and its clinical use as an anticoagulant. His published work includes the original paper (with F. G. Banting) on insulin, "The internal secretion of the pancreas" (*J. Lab. clin. Med.* 1922, 7, 251), and (with D. W. G. Murray, L. B. Jaques & T. S. Perrett) "Heparin and vascular occlusion" (*Canad. med. Ass. J.* 1936, 35, 621). He is joint author (with N. B. Taylor) of *The physiological basis of medical practice: a text in applied physiology* (6th ed., 1955).

DAME HARRIETTE CHICK is a graduate of the University of London, an 1851 Exhibitioner, Fellow of University College, London, and a member of the Scientific Staff of the Lister Institute of Preventive Medicine, London. Her early researches in bacteriology and biochemistry dealt with the mechanics of the disinfection process and the heat coagulation of proteins. During the First World War her group at the Lister Institute, in response to the nutritional needs of our armies serving abroad, studied the quantitative distribution in foods of the newly discovered vitamins. Dr Chick was entrusted subsequently with a scientific mission sent by the Accessory Food Factors Committee to post-war Vienna to study the nutritional deficiency diseases prevalent in that city, where much of the work of the mission was devoted to problems of the aetiology of rickets. Her later work has included investigation of the physiological action of different members of the B group of vitamins, of the nutritive value of proteins (especially of wheat and maize) and of the aetiology of pellagra. Dr Chick has acted as Secretary of the Accessory Food Factors Committee (of the Lister Institute and Medical Research Council) from the time of its inception intil 1947.

DR M. E. COATES is a member of the staff of the Department of Nutrition at the National Institute for Research in Dairying, Shinfield, Reading, a position which she has held for the past ten years. Before this she was Research Assistant in the Biochemical Department of Glaxo Laboratories Ltd., Greenford, Middlesex. Her main interests have been in the problems of nutrition, particularly the importance of vitamins in animal nutrition. Her present work is concerned with the mechanism of action of antibiotics in promoting animal growth, with the role of vitamin B_{12} and possible unidentified growth factors, and with methods of biological assay of vitamins. Her publications have included a number of papers on each of these topics.

DR C. E. DALGLIESH is a lecturer in biochemistry in the Postgraduate Medical School of London. He graduated from the University of Cambridge in 1942 and continued at Cambridge to do research on the synthesis of polycyclic compounds, amino acids and polypeptides. He was elected a Fellow of Trinity College in 1945, and in 1946–47 held a Commonwealth Fund Fellowship at Harvard University. In 1949 he joined Dr A. Neuberger's team at the National Institute for Medical Research, London, and in 1952 took up his present appointment. His principal interests are in intermediary metabolism, particularly of the aromatic amino acids and proteins, and more widely in the biochemistry of man.

DR HONOR B. FELL is a graduate of Edinburgh University, and came to Cambridge in 1923 as Research Assistant to the late Dr T. S. P. Strangeways under the Medical Research Council. She has held in succession a Beit Memorial Fellowship and two Royal Society Fellowships—the Messel Research Fellowship, and the Foulerton Research Fellowship, which she still holds. Dr Fell was appointed Director of the Strangeways Research Laboratory, Cambridge, in 1927, and was elected a Fellow of the Royal Society in 1952. She has written a number of papers on various aspects of tissue culture, and is now studying the direct action of hormones and vitamins on organ cultures of various types.

DR T. W. GOODWIN, after graduating at the University of Liverpool at the beginning of the Second World War, remained there to work with Professor R. A. Morton for the Ministry of Food on various problems concerned with vitamin A metabolism, especially in relation to human requirements. When the war ended he was appointed Lecturer in Biochemistry in the University of Liverpool and in 1949 he was promoted to Senior Lecturer, a post which he now holds. During the immediate post-war period he worked with Professor Morton on a number of problems, including the identification of retinene as vitamin A aldehyde, and the demonstration of the intestinal wall as the main site of the conversion of carotene into vitamin A. More recently, his interests have turned to problems of biosynthesis, and he is at present working on the biosynthesis of carotenoids and riboflavin in micro-organisms. Dr Goodwin has published numerous papers (in collaboration with Professor Morton and others) in the *Biochemical Journal*, and he is author of *The comparative biochemistry of carotenoids* (1952).

SIR CHARLES HARINGTON has been Director of the National Institute for Medical Research, London, since 1942. Prior to this, he was Professor of Chemical Pathology in the University of London from 1931 to 1942, and Director of the Graham Medical Research Laboratories, University College Hospital Medical School, London, from 1937 to 1942. He was elected a Fellow of the Royal Society in 1931, and was Croonian Lecturer of the Royal Society in 1944, when the title of his lecture was "Thyroxine: its biosynthesis and its immuno-chemistry". Sir Charles Harington was a member of the Medical Research Council from 1938 to 1942, and editor of the *Biochemical Journal* from 1930 to 1942. He has published numerous papers on chemical and biochemical subjects, mainly in the *Biochemical Journal*.

DR LESLIE J. HARRIS has been Director of the Dunn Nutritional Laboratory (University of Cambridge and Medical Research Council) since its inception in 1927. He is a joint founder and, at the moment, President of the Nutrition Society. He is also Chairman of the Biological Methods Group of the Society for Analytical Chemistry, Secretary-General of the International Union of Nutritional Sciences, and a joint editor of the *British Journal of Nutrition*. He was awarded a Leblanc Medal by Manchester University in 1921, and the Meldola Medal by the Royal Institute of Chemistry in 1925. He is the author of several standard books on vitamins, some of which have run into numerous English and foreign editions. He has also contributed authoritative reviews on vitamins to the *British encyclopaedia of medical practice* (2nd ed., 1952), to *Chambers's encyclopaedia* (1950) and to *Thorpe's dictionary of applied chemistry* (4th ed., 1954), etc. His research investigations, covering a wide field in biochemistry and physical chemistry, have been published in the *Proceedings of the Royal Society*, *Philosophical Transactions of the Royal Society*, *Biochemical Journal*, *British Journal of Nutrition*, *Lancet* and elsewhere.

DR W. I. M. HOLMAN has been a member of the Scientific Staff of the Medical Research Council since 1947, when he left the Rowett Research Institute, Aberdeen, to carry out the vitamin work in the nutritional investigations which were at that time being made by Professor R. A. McCance's unit in Germany. He later spent a short period at the South African Institute for Medical Research, Johannesburg, working on nicotinic acid metabolism in relation to pellagra. Since 1950 he has worked at the Department of Experimental Medicine, Cambridge. His publications have been principally concerned with the biochemistry of B vitamins, and include a number of papers on the metabolism of nicotinic acid and on the pyridone which is an important metabolite of nicotinic acid in man.

MISS E. M. HUME is a graduate of Cambridge University where she did research in botany. She became Lecturer in Botany in the University of Cape Town in 1914. She returned to England during the First World War and began to work on vitamins under Dame Harriette Chick at the Lister Institute of Preventive Medicine, London, where she has remained ever since. The work was concerned chiefly with a search for foods which would protect the troops against beriberi and scurvy, and for suitable antiscorbutic supplements for infants. She was later appointed a member of the External Scientific Staff of the Medical Research Council. She went with the mission sent by the Lister Institute and the Medical Research Council to study rickets in Vienna in 1919–22, and, as Secretary of the Vitamin A Subcommittee, was closely associated with the work of the Accessory Food Factors Committee. She organized and took part in its work on vitamin standardization, and in the experiment to deprive human volunteers of vitamin A which was carried out at Sheffield in the Second World War.

DR S. K. KON has previously contributed to the Bulletin (*Brit. med. Bull.* 1947, 5, 170) an article entitled "A survey of the place of cow's milk in the human diet and of the effect of processing on its nutritive value", with which a biographical note was published.

PROFESSOR COLIN C. LUCAS graduated from the University of British Columbia and, since 1934, has worked in the Banting and Best Department of Medical Research, University of Toronto. From 1929 to 1934, he was Professor of Chemistry at Brandon College, Brandon, Manitoba. He has examined the essential oil of the yellow cedar (*Chamaecyparis nootkatensis*), and has done chemical oceanography on the Pacific coast of Canada. In Toronto he worked with the late Sir Frederick Banting and with Dr E. J.

NOTES ON CONTRIBUTORS

King on biochemical aspects of silicosis; on glutathionine and cystine; and on the fractionation of protein hydrolysates. He has developed a microchemical method for detecting lead in urine, which permits prompt diagnosis of lead poisoning in infants and, with Professor C. H. Best, Dr Jessie H. Ridout and others, he has studied lipotropic phenomena. He is also interested in dietary protein in the prevention of liver damage, and has studied biochemical phases of chemotherapy of the sulpha drugs. Professor Lucas's publications, with other authors, include: "The chemical nature of royal jelly" (*Biochem. J.* 1940, **34**, 1155); "Effect of dietary proteins and amino acids on liver fat" (*J. biol. Chem.* 1945, **160**, 505); and "The lipotropic activity of protein" (*Canad. J. Biochem. Physiol.* 1955, **33**, 25).

PROFESSOR R. A. MCCANCE, in collaboration with Dr E. M. Widdowson, has previously contributed an article to the Bulletin (*Brit. med. Bull.* 1951, **7**, 297) on the composition of the body, with which a biographical note was published.

DR L. W. MAPSON is a principal scientific officer at the Low Temperature Research Station, University of Cambridge and Department of Scientific and Industrial Research. During the Second World War, he was seconded to the Dunn Nutritional Laboratory (University of Cambridge and Medical Research Council), for research into the effect of modern dehydration processes on the nutritive value of foods. During the post-war period Dr Mapson has been engaged in biochemical research into (i) the biological mechanisms whereby vitamin C is synthesized in plant and animal tissue (*Biochem. J.* 1953, **56**, 1, 21; 1955, **59**, ix); (ii) the conditions which determine its stability in the cell; and (iii) its role with glutathione as a component of a respiratory enzyme system in plant cells (*Biochem. J.* 1951, **49**, 592; 1956, **62**, 248).

DR THOMAS MOORE has been Deputy Director of the Dunn Nutritional Laboratory (University of Cambridge and Medical Research Council) since its inception in 1927. He has been engaged in research work on nutrition, mainly on vitamins A and E, and has been a member of the Medical Research Council's Subcommittees on these vitamins.

PROFESSOR R. A. MORTON has since 1944 been Johnston Professor of Biochemistry in the University of Liverpool, where he was formerly Lecturer in Inorganic and Physical Chemistry, and Lecturer in Spectroscopy. In 1930 he was Visiting Professor at the Ohio State University, Columbus, Ohio; and was awarded the Meldola Medal by the Royal Institute of Chemistry in the same year. During the Second World War, he carried out work for the Ministry of Food on vitamin oils and margarine, and was also concerned with the Medical Research Council's experiment on the human requirement of vitamin A, and with chemical defence and fire research. His post-war research included the application of spectroscopy to chemical and biochemical problems; investigations on vitamins A and carotenoids; and studies on the chemistry of vision. Professor Morton's work in progress is concerned with the systemic mode of action of vitamin A, and with derangements in sterol metabolism in various species. His publications include numerous papers in scientific journals, and *The application of absorption spectra to the study of vitamins, hormones and coenzymes* (2nd ed., 1942).

SIR RUDOLPH PETERS is now a member of the Scientific Staff of the Agricultural Research Council at Babraham Hall, Cambridge. From 1923 until his retirement in 1954, he was Whitley Professor of Biochemistry in the University of Oxford. During the early part of his career, he was a pupil of both the late Sir Joseph Barcroft and the late Sir Frederick Gowland Hopkins. After work with Sir Joseph Barcroft on the specific oxygen capacity of haemoglobin, he turned his attention to vitamin problems, and his name is particularly associated with work on the vitamin B complex; lately he has worked with colleagues upon arsenical problems (British anti-lewisite), and upon the cattle poison fluoroacetate. Sir Rudolph Peters was awarded the Royal Medal of the Royal Society in 1949. He is also an honorary or foreign member of various scientific societies and academies, including the Royal Netherlands Academy of Science and Letters, the Accademia Nazionale del Lincei, Rome, and the American Academy of Arts and Sciences.

MRS ANTOINETTE PIRIE is Reader in Ophthalmology, University of Oxford, and Head of the Nuffield Laboratory of Ophthalmology, Oxford. She is a biochemist, trained in Cambridge under the late Sir Frederick Gowland Hopkins. Her interest in the biochemistry of the eye began during the Second World War through collaboration with Miss Ida Mann. She has investigated the effect of vitamin deficiencies on the eye, with particular reference to the relation between biochemical and pathological changes, and her recent work has included studies of the chemistry and structure of the vitreous body and biochemical changes in the lens during formation of cataract. Mrs Pirie's published work is in the *Biochemical Journal*, the *British Journal of Ophthalmology* and the *British Journal of Nutrition*; her book on the *Biochemistry of the eye* is being published shortly.

PROFESSOR B. S. PLATT is Director of the Human Nutrition Research Unit at the Medical Research Council Laboratories at Hampstead, London, and Head of the Department of Human Nutrition and Director of the Applied Nutrition Unit at the London School of Hygiene and Tropical Medicine. He was Chairman of the joint Food and Agriculture Organization and World Health Organization Expert Committee on Nutrition which met in Geneva in October–November 1954; and he was President of the 2nd Inter-African (C.C.T.A.) Conference on Nutrition, held under the auspices of the Commission for Technical Co-operation in Africa South of the Sahara, at Fajara, Gambia, 19–27 November 1952. Professor Platt has contributed three articles to earlier numbers of the Bulletin: "Aspects of nutritional research" (*Brit. med. Bull.* 1944, **2**, 204); "Notes on some cutaneous manifestations of dietary deficiencies" (*Brit. med. Bull.* 1945, **3**, 179); "Nutritional comparison of human and cow's milk for infant-feeding" (*Brit. med. Bull.* 1947, **5**, 177), and biographical notes were published with these.

DR J. W. G. PORTER was educated at Emmanuel College, Cambridge. After four years' postgraduate research in the Department of Organic Chemistry at Cambridge he joined the Department of Nutrition at the National Institute for Research in Dairying, Shinfield, Reading, where he is working on the role of the intestinal microflora in the vitamin metabolism of the pre-ruminant and ruminant cattle. Dr Porter is interested in the biochemical aspects of problems in animal nutrition, particularly those concerned with the water-soluble vitamins. He has published papers on the mode of action of antibiotics, on factors related to vitamin B_{12}, and on the synthesis of vitamins in the rumen.

DR JESSIE H. RIDOUT is a graduate of the University of Toronto. Since 1942 she has been Research Associate in the Banting and Best Department of Medical Research, University of Toronto; for some years before this she was Research Assistant in the Connaught Medical Research Laboratories, University of Toronto. From 1939 to 1942, she worked with the Canadian Red Cross Society on the preparation of human serum for the use of the armed forces. Dr Ridout has studied, with Professor C. H. Best, the metabolism of exercise in trained and untrained athletes and, later, some phases of carbohydrate metabolism. Since 1932 she has been investigating nutritional problems (lipotropic agents and cholesterol metabolism). She is the author, ointly with others, of a number of papers, for example: *Annu. Rev. Biochem.* 1939, **8**, 349; *Amer. J. Path.* 1951, **27**, 951; *Brit. med. J.* 1955, **1**, 1439.

DR H. M. SINCLAIR is Vice-President, Fellow and Lecturer in Physiology and Biochemistry of Magdalen College, Oxford, and Reader in Human Nutrition in the University. During the Second World War he directed nutritional surveys for the Ministry of Health; just before the end of the War he took a team to the Netherlands at the invitation of the Dutch Government to study the famine there, and then organized nutritional surveys in Germany as Honorary Nutritional Consultant to the Control Commission. He was awarded the Dutch Order of Orange-Nassau and the United States Medal of Freedom with Silver Palm. Dr Sinclair's work has been mainly concerned with fundamental problems of human nutrition and metabolism, including methods for assessing human nutriture, and the relation of nutrition to disorders of the skin and nervous system. He is interested in international applications of nutritional science (see, for example, *Nutrition and health* (1953), written with Sir Robert McCarrison); and in education (see "The teaching of nutrition" in *Proc. 1st World Congr. Med. Educ.*, 1953, p. 717). He has formed one of the best private collections of old medical books in Britain, being interested in medical history, and is the author, with A. H. T. Robb-Smith, of *A short history of anatomical teaching in Oxford* (1950). In 1951 he delivered the Cutter Lecture at Harvard University.

DR E. LESTER SMITH is now Senior Biochemist at Glaxo Laboratories Ltd., Greenford, Middlesex, having served the Company in various capacities for nearly 30 years. He has worked on research and development of a range of fine chemicals, and vitamins of both natural and synthetic origin. He was associated with the early efforts to produce penicillin in Great Britain during the Second World War, and worked on the biogenesis of the antibiotic and methods for its extraction and crystallization. An increasing interest in biochemistry and fundamental research led in 1948 to the isolation of vitamin B_{12}, independently of and very shortly after its first isolation in the USA. Dr Lester Smith has since worked on the chemistry of vitamin B_{12} and its analogues, several of which were first isolated in his laboratory. In the course of his work he has made contributions to tracer techniques, partition chromatography and paper electrophoresis, and has published about 80 original papers. In 1954 he was awarded the Gold Medal in Therapeutics of the Worshipful Society of Apothecaries, and in October 1955 gave the Lister Memorial Lecture of the Society of Chemical Industry. An article by Dr Lester Smith, entitled "Radioactive penicillin and vitamin B_{12}", appeared in *British Medical Bulletin*, 1952, **8**, 203.

PROFESSOR L. J. WITTS was Assistant Physician to Guy's Hospital, London, from 1929 to 1935, and Professor of Medicine at St Bartholomew's Hospital, London, from 1935 to 1938. He is now Nuffield Professor of Clinical Medicine in the University of Oxford. He has twice been a member of the Medical Research Council, and he is a member of the Scientific Advisory Committee of the Lady Tata Memorial Trust for research on leukaemia. He has also been Secretary and Treasurer of the Association of Physicians of Great Britain and Ireland, and is an honorary member of the Danish Society of Internal Medicine and the American Association of Physicians. Professor Witts gave the Goulstonian Lectures on anaemia in 1932 and the Frederick Price Lecture on the survival of the red cell in 1950. His more recent research work has been concerned with the production of pernicious anaemia by intestinal *culs-de-sac*, and the application of radioactive iron and radioactive vitamin B_{12} to the study of anaemia. He has also conducted trials of cortisone in ulcerative colitis and blood disorders.

Book Reviews

Infant Nutrition in the Subtropics and Tropics

D. B. Jelliffe. Geneva: World Health Organization, 1955. (World Health Organization Monograph Series, No. 29.) 237 pages; plates. 24 × 16 cm. £1 5s., $5.00, or Sw.fr. 15.

World Population and Resources: A Report by PEP

London: PEP (Political and Economic Planning), 1955. xxxvii + 339 pages; figures. 22 × 14 cm. £1 10s.

Both the books under review have one aim in common, i.e., to define a problem. The author of the book on infant nutrition hopes that the results of his study will assist paediatricians and other workers concerned with maternal and child health to become aware of their problems and

> thereby to shorten the 'phase of bewilderment', which at present appears to be inevitable before it becomes realized that methods used successfully in the countries of training are often absolutely unsuitable for less technically developed tropical regions, where a very different cultural and dietetic background may exist.

The objects of Political and Economic Planning (PEP) are to study questions on which public thought and discussion are needed, and to present both the facts themselves, and the conclusions which they seem to point, in an objective and readable way.

> The aim is to provide a clear and accurate account of all the main elements of the problem and to draw attention to the likely consequences of possible lines of action as a basis for recommendations on policy.

To the best of my knowledge there is no other book that covers the same ground as the World Health Organization (WHO) monograph; it is a unique and a much-needed treatise. It contains a record of the author's personal surveys of infant feeding practices in many countries. A variety of nutritional diseases which are prevalent amongst infants in the tropics and subtropics are described, and measures which are employed, or might be introduced, to avoid nutritional disease, ill health and death during the rearing of infants are given. The author includes a chapter written in collaboration with Dr Anne Burgess, Consultant to the Central Council for Health Education, London, on "Nutrition education in the maternal and child health (MCH) centre", rightly stressing the need on the part of the MCH worker of a

> . . . much more imaginative approach than has been usual in the past, with its tendency to rely mainly on didactic teaching, and an occasional demonstration in the clinic or the home.

I can only add that no one interested in the problem can afford to miss reading this book; few will fail to find something of interest and profit; and I am very glad to find someone supporting my own views on the pre-eminence of the mother as a source of nourishment for her own infant. And, of course, the fact is recognized that the mother must be properly fed to enable her to feed her baby well enough and for a sufficiently long period so that the child is gradually accustomed to a variety of suitable foods and ultimately achieves nutritional independence of his mother. These are simple and obvious principles—yet so often apparently completely overlooked.

By contrast, there have been many books written on the growth of the world's population in relation to the resources of food; and much has also been written on the world needs of supply of energy, minerals and other commodities, in the future. The most difficult problem of all, according to the PEP report, is to increase the production of food in the decades ahead sufficiently to allow the standard of nutrition of the underfed millions to be raised and to provide for the additional mouths to be fed at the present—or possibly even greater than the present—growth in size of human populations. The report's main conclusion is that ". . . . in future death-control should proceed not in isolation but hand-in-hand with birth-control".

The "conspiracy of silence" about the regulation of the size of populations must be broken if this most formidable of the world's urgent and practical problems is to be tackled—as indeed it must be tackled. It is a problem which may well be identical with the problem of causation of war itself. The PEP report provides quantitative data, with illustrations drawn from a number of typical communities, to enable people to examine and to discuss the matter.

I have seized the opportunity to put these two books together as I have recommended "Maternal and child-welfare work . . . as a first step in promoting social and economic development among unsophisticated people"; I have argued the case for this in relation to the main problem of "relating resources to the size of the population" (*Lancet*, 1954, **2**, 929—reprinted in *Community Development Bulletin*, 1955, **6**, 50). Death control applied at an early age is likely to be the biggest factor in increasing still further the present unprecedentedly high rate of increase of the world's population, since in many tropical communities death-rates in young children are appallingly high, as many as half the babies born dying before maturity. Birth control cannot be adopted until a measure of education and economic development is achieved; what better place to begin than in the welfare clinic, and who is more concerned in it than the mother?

B. S. Platt

Studies on the Nutritive Value of Bread and on the Effect of Variations in the Extraction Rate of Flour on the Growth of Undernourished Children

E. M. Widdowson & R. A. McCance. London: Her Majesty's Stationery Office, 1954. (Medical Research Council Special Report Series No. 287.) viii + 137 pages; figures. 24 × 15 cm. 8s. 6d.

In 1946, undernutrition was prevalent in Germany. At Wuppertal, in the then British zone, children at two orphanages were clinically examined. Their diets had been insufficient, and in consequence the children were below the accepted standards in weight and height. It was these undernourished children who were made the subject of the studies described in the above-mentioned report.

The principal object of the investigation was to compare the nutritive value of different types of bread, particularly to test the effect of variations in the so-called "extraction rates" of the flours used—an important point in national food policies. The trials continued for a year, during which time the children were restricted to the various experimental diets. The work was carried out under the direction of Professor R. A. McCance and Miss E. M. Widdowson of the Department of Experimental Medicine at Cambridge University, and was under the auspices of the Medical Research Council.

It is impossible to praise too highly the care and thoroughness with which the experiments were done. The published account is a model of scientific reporting: careful, cautious and exhaustively documented. It deserves to be, as it will be, consulted and studied by nutritionists in all regions of the globe.

Unfortunately the authors were unable to demonstrate whether the different types of bread were equally nutritious or not, for the children grew equally well on each. In fact they grew considerably better than normal children of the same age. The explanation, no doubt, is twofold. Firstly, the previous diet had been so poor that what was observed was not normal growth at all but a "recovery spurt"—making up for lost time. Secondly, the basal diet used in the trials, mostly of vegetable origin, was so complete in various nutrients, such as vitamin B_1 and nicotinamide, that any deficiency in the bread would have been obscured.

Dr Widdowson and Professor McCance are themselves the first to emphasize that the greatest caution must be used in attempting to deduce from their results any conclusions of more general applicability. As the Medical Research Council say in their foreword:

> The conclusions drawn must be restricted to the conditions under which the scientific evidence was obtained and cannot justifiably be applied to the needs of populations in very different states of nutrition and with widely varying dietary habits.

Leslie J. Harris

Vitamins in Theory & Practice

Leslie J. Harris. 4th ed. Cambridge: University Press, 1955. xxii + 366 pages; 105 illustrations. 21 × 15 cm. £1 15s.

The aim of the author has been to provide a book on vitamins which will be of value not only to scientific and medical students, but also to dieticians, social-welfare workers and the layman who is interested in nutrition. The author has amply succeeded in this aim and produced a book which cannot fail to hold the attention and interest of the reader whilst at the same time providing the student with the fundamental knowledge upon which the science of nutrition is based.

The first chapter is devoted to the history of the discovery of vitamins; succeeding chapters are devoted to each vitamin in turn, the vitamins being some score in number. For each of these the clinical picture of the disease associated with the deficiency of the vitamin is described, and the text is amplified by excellent illustrations of the symptoms of deficiency diseases in man and animals. The chemistry of each vitamin is dealt with, and the methods for its assay critically examined. Finally, the distribution and the stabilization of the vitamin in foods, its mode of action in vivo, and methods of assessing the level of nutrition, are reviewed. The author's aim to mention every major fact of importance about the vitamins does in truth seem to have been achieved.

The author concludes with a chapter on dietetics which will prove of great interest to the lay reader, for it disposes of the fallacy that the health of the nation was all right in the "good old days" before vitamins were heard of and shows the correlation between the improvement in the nation's dietary and its health during the last 25 to 50 years.

As an introduction to the science of nutrition the book cannot be praised too highly, for it not only captures the imagination of the reader, but it brings home to the student the practical significance of the results obtained in the laboratory.

L. W. Mapson

The Biochemistry of Vitamin B_{12}

Organized and edited by *R. T. Williams.* Cambridge: University Press, 1955. (Biochemical Society Symposia No. 13.) 123 pages; figures. 25 × 16 cm. Cloth binding: £1; paper binding: 13s. 6d.

The isolation of vitamin B_{12} in 1948 by two independent groups of workers, one in the USA and another in Great Britain, has led to the identification of this vitamin as the anti-pernicious anaemia factor, long known to be present in the liver, and stimulated a vast amount of research into its role in animal and microbial metabolism.

This publication of the Biochemical Society gives in seven articles a fairly comprehensive view of our present knowledge concerning the chemistry and biochemistry of vitamin B_{12} and of allied substances. Sir Alexander Todd, whose work contributed so much to the recent identification of the structure of vitamin B_{12}, wrote the Introduction. E. Lester Smith, who with his colleagues at Glaxo Laboratories Ltd. was responsible for the isolation in Great Britain of vitamin B_{12} from liver, discusses briefly in his article the methods of extraction of the vitamin from various sources and gives a more detailed account of the steps which led to the identification of the constituent chemical groupings and their linkages in this very complex vitamin. Dorothy Hodgkin's contribution to the discussion of Lester Smith's article foreshadowed the elucidation of the complete structure of vitamin B_{12} by *x*-ray crystallography, and was published shortly after the symposium was held (*Nature, London,* 1955, **176,** 325). The article by S. K. Kon on factors related to vitamin B_{12} sets out very clearly their interrelationships. As there are at present about ten such substances known and still referred to by letters of the alphabet, a clear statement on these is particularly welcome. Now that the chemical structure of vitamin B_{12} has been established, both by the *x*-ray crystallographic work of Dorothy Hodgkin and by the chemical approach of Sir Alexander Todd's team, there is a hope that systematic chemical nomenclature may be applied to these various factors. M. E. Coates & J. E. Ford present an account of the methods (chemical, biological and microbiological) of assaying vitamin B_{12} and related factors. The preparation of vitamin B_{12} labelled with either of the two radioactive isotopes of cobalt, ^{60}Co and ^{58}Co, assisted greatly the study of the absorption and excretion of vitamin B_{12} in man and the factors influencing these processes. D. L. Mollin & S. J. Baker give an account of such studies on normal subjects and on patients with pernicious and other megaloblastic anaemias. Since Castle's intrinsic factor is essential for the absorption of vitamin B_{12}, it is appropriate that an article by A. L. Latner on this factor is included in the series. The last two articles in the volume, one by H. R. V. Arnstein and another by June Lascelles & M. J. Cross, summarize the present knowledge concerning the function of vitamin B_{12} and related substances in the metabolism of animals and micro-organisms respectively.

This symposium should appeal by its broad scope to chemists, biochemists, microbiologists, nutritionists, and haematologists as well as clinicians. It is the first time that this series of publications of the Biochemical Society is offered with a hard binding as well as the former paper binding. It is a pity that the reading of proofs has not been as careful as one usually associates with publications of the Cambridge University Press.

G. Popják

Pharmacopoeia Internationalis, Volume II

World Health Organization. Geneva: World Health Organization, 1955. xx + 350 pages. 25 × 16 cm. £1 15s.

The International Pharmacopoeia has been prepared with a view to establishing recommended international standards and nomenclature for pharmaceutical preparations in general use. Volume I (published in English and French in 1951, and in Spanish in 1954) was reviewed in the Bulletin (*Brit. med. Bull.* 1952, **8,** 271). Volume II, now available in English and French and soon to appear in Spanish, completes the task begun in 1937 under the auspices of the League of Nations Health Organization, and carried on since 1947 by the World Health Organization. Among the 217 monographs are those on drugs—such as antibiotics—that have been introduced into therapeutics during the past few years. There are monographs on some recently introduced synthetic substances, as well as on compressed tablets, sterile injections and tinctures of substances described in volume I. Appendices include descriptions of relevant methods of assay for some of the substances; a table giving the usual and maximal doses for adults of most of the drugs included, and a table of usual daily doses for children of certain drugs. An annex on solutions of cardiolipin and lecithin for serological tests has been added, in view of the increasing use of these substances in such tests. Volume II is provided with a detailed alphabetical index designed to cover both volumes I and II.

O. M. H.

The Extra Pharmacopoeia (Martindale), Volume II

Published by direction of the *Council of the Pharmaceutical Society of Great Britain:* 23rd ed. London: Pharmaceutical Press, 1955. xxxi + 1501 pages. 19 × 13 cm. £2 17s. 6d.

The two volumes of the *Extra Pharmacopoeia* are intended as companion volumes to the *British Pharmacopoeia 1953* and the *British Pharmaceutical Codex 1954.* Volume I of the 23rd edition of the *Extra Pharmacopoeia* was published in 1952, and about one-quarter of volume II of this edition comprises analytical addenda to the chemicals and materia medica in the first volume. Other sections cover a variety of subjects, ranging from the microbiological assay of vitamins to proprietary medicines; the sections on clinical biochemistry and haematology have been greatly expanded.

The most striking examples of advances in nutritional research since the publication of the previous edition are provided in the section on vitamins. The development of physico-chemical methods for estimating vitamins, which are replacing biological assays, has resulted in the accumulation of a vast amount of information. For each vitamin, the *Extra Pharmacopoeia* has a section on nomenclature, source, properties and functions, and methods of assay, and there are additional sections on human requirements, and on symptoms of deficiency. Problems of standardization of vitamins in foods and pharmaceutical products, and the supplementation of diets with vitamins, are dealt with briefly.

O. M. H.

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Scientific Editor: *C. S. Hallpike*

Neuro-Otology : Introduction

The present number of the Bulletin gives clear evidence of the active state of otological research and its present fruitfulness. The unusual difficulty of experimentation on the ear and labyrinth delayed the attack on some otological problems until recent technical advances brought them within the range of examination. The advances during the past twenty-five years in knowledge in this field have been most remarkable, and the pace at present shows no sign of slackening. The present series of papers furnishes an example of the fact that in the study of the ear many disciplines meet. Physiology, physics, pathology, clinical investigation, histology and biochemistry are essential partners in the extension of understanding of these mechanical sense organs, the most highly organized we know. Electronic devices have made a great contribution not only by providing the means to explore the electrical events in nerves and sense organs, but also by providing the tools to measure and produce accurately graded sounds and vibrations and to control rotations used as stimuli. It would be hard to over-estimate the importance of the general adoption of quantitative methods in both laboratory and clinical studies of ear and labyrinth.

During the last quarter-century great changes have taken place in the explanations afforded for many auditory phenomena. It is a hundred years since Helmholtz formulated his theory of hearing, and it has been of enormous value to focus quantitative ideas on to the cochlea. However, the increase of knowledge of the neural processes in the conducting pathway, and detailed examination of mechanical and electrical events in the cochlea, have now made the theory of mechanical resonance of stretched fibres quite untenable as a basis for interpretation of sound analysis by the ear, for with pure tones the highly localized disturbance of the basilar membrane required to restrict stimulation to a few nerve fibres does not in fact occur. Nevertheless, the subjective phenomena of hearing on which Helmholtz based his theory had shown that the cochlea with its neural connexions to the cortex does carry out analysis very like the separation of Fourier components, which can be done by a system of mechanical resonators. It is now clear that this is in part due to properties of neurones in the pathway, with complex interaction occurring in the nuclei between the activity of the primary and secondary neurones. Studies of action potentials in the primary neurones and in the nuclei enable the analytical processes in the cochlea itself, and in the pathway, to be separated; this helps our understanding not only of hearing, but of the transformations that appear common to many sensory pathways within the

central nervous system—for example, those for impulses from the skin, where the principle of maximal stimulation has long been recognized and attributed to interactions such as are now being observed to occur in the auditory pathway.

But no less important are the mechanical analytical functions of the cochlea. It has become clear that an orderly arrangement in space of the representation of hair cells along the basilar membrane is preserved throughout the neural pathway and in the auditory cortex. It is also established beyond reasonable doubt that the fluid disturbance travels up the cochlea for a distance which is an inverse function of the frequency of the incident sound wave. This disturbance of endolymph and perilymph is by no means simple, and the constants determining it are not yet all clearly defined; but it seems likely that not only the graded resistance to displacement of the basilar membrane but also the properties of a hydraulic column in acceleration can account for much of what is observed.

Just over one hundred years have elapsed since Reissner described his membrane and discovered the cochlear duct, showing two separate fluid channels in the cochlea as in the labyrinth. Recent work shows the striking differences in composition between endolymph and perilymph, particularly in potassium/sodium ratio, and carries with it implications of osmotic work to be done in endolymph production, and of electrical gradients where it is separated from body fluids of normal composition by semi-permeable membranes. The recent demonstration of steady potentials of 50–100 millivolts maintained across Reissner's membrane, and between endolymph and structures on the basilar membrane, is consistent with the ionic differences found.

Recent analysis of the origin of microphonic potentials shows this to be sited close to the hair cells and brings back into prominence the theories of twenty years ago, which suggested that the cochlear microphonics arise by modulation of a local potential source. It does not seem unlikely that hair cells may perform some such function which would make it easier to understand the extraordinarily high sensitivity achieved in the ear. In other mechanical sense organs, generator potentials have been found to initiate the rhythmic discharge of impulses. In the ear, such a mechanism would appear to be developed far beyond anything known elsewhere.

Since the days of boiler-maker's disease, clinical observations of abnormal states of the organ of hearing have played a large part in elucidating the physiology of the ear, and the increasing use of quantitative clinical methods has maintained this important contribution.

The mechanical problems of orientation in the gravitational field and control of motion are much the same for the whole animal kingdom, with some handicap to those submerged in water which lack asymmetric stimuli to the body wall and clear visual orientation to assist in the process. The sense organs of the labyrinth are highly developed in animals far down the evolutionary scale, and in general have close structural affinities with those of the higher mammals and man, but are much more accessible to experiment. Analysis of these has given precision to theories of the action of the semicircular canals and otolithic maculae. In man, quantitative methods of analysis of reactions to displacement, and to rotation, have enabled the details of the action of the labyrinth also to be defined in exact terms.

Vestibular tests are valuable tools to the clinicians other than otologists. The close control exercised by these sense organs on the whole musculature of the mammal is of paramount importance in many practical situations. At present it appears probable that much of this control may be mediated via the small efferent nerve fibres to muscle spindles, so that the vestibular sense organs may control the mechanical sensitivity to stretch of muscle sense organs distributed throughout the body.

The devastating effects on the central nervous system of the vertigo and disorientation that can occur with abnormal stimulation of the labyrinth becomes an important practical problem in aircraft where complex combinations of linear and angular accelerations may occur which the labyrinth is unable to resolve. At present much training has to be devoted to overcoming the normal reactions in pilots to these abnormal stimuli and to ensure that gyroscopic instruments are believed and the impulses from the labyrinth discounted. In the contribution upon the investigation of vestibular function[1] it is of particular interest to note the reference made to a recent finding that, whereas no habituation occurs in man or animals to the response to caloric stimulation of a single semicircular canal, such habituation does occur with rotational stimuli that involve the normal balance of stimulation of at least two canals during the onset and cessation of angular motion. This suggests that habituation, which is clearly an elaborate neural process, may become susceptible to quantitative treatment.

The present series of papers covers problems throughout the field of otology and shows what a close liaison exists here between laboratory and clinic, to the great advantage of both. The stimulus to research in this field by the formation in 1944 by the Medical Research Council of their Otological Research Unit, and the very active interest taken by the Medical Research Council in the otological health and hazards of the nation, have played an important part in the great advances achieved and in keeping Great Britain in the forefront of research in this field.

Bryan Matthews

[1] Cawthorne, Dix, Hallpike & Hood, p. 138.—ED.

The subject of this symposium was suggested by Dr E. A. Carmichael who also acted as Chairman of the planning committee. We are much indebted for all his help and advice throughout.

NOTES ON THE TECHNIQUE OF TEMPORAL BONE MICROTOMY

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A Dimensions, form, sharpening and application of knives for temporal bone microtomy
- 1 Length of knife edge and angle of cutting stroke
- 2 The angle of the cutting stroke (α)
- 3 The cutting angle (Y)
- 4 Sharpening
- 5 Control of the cutting angle (Y)
- 6 Microscopic examination of the knife edge

B Metallurgical characteristics of knives for temporal bone microtomy

C Nitrocellulose : its manufacture, physico-chemical characteristics, and use for temporal bone microtomy
- 1 Manufacture of nitrocellulose
  - *a* Nitration
  - *b* Finishing
- 2 Physico-chemical characteristics of nitrocellulose
- 3 Use of nitrocellulose for temporal bone microtomy

References

The adult human temporal bone, when shaped for histological examination, takes the form of a block of very dense bone, having the following approximate dimensions: length 40 mm., breadth 25 mm., depth 25 mm. Within it lies the peripheral apparatus of hearing and equilibrium, and the study of its structure requires the preparation of serial histological sections, a task attended by difficulties which are considerable and well known. Nitrocellulose is accepted as the most suitable embedding medium, and the usual section thickness is 25 μ.

In the present paper a brief description is given of some of the technical methods developed since 1940 in the Histological Department of the Otological Research Unit of the Medical Research Council, for the cutting of large numbers of sections of this hard material, together with an outline of the physical principles upon which they are based. The subject falls into three divisions, which are concerned respectively with:

i. Dimensions, form, sharpening and application of knives for temporal bone microtomy;
ii. their metallurgical characteristics;
iii. the manufacture and physico-chemical characteristics of nitrocellulose (celloidin) and its use for temporal bone microtomy.

A. DIMENSIONS, FORM, SHARPENING AND APPLICATION OF KNIVES FOR TEMPORAL BONE MICROTOMY

We have throughout used a Leitz base-sledge microtome, modified in a manner which makes possible the use of a new design of knife. This is held with great rigidity and freedom from internal stresses by means of the knife holder shown in fig. 1, mounted upon a massive casting. The temporal bone block is mounted upon the sliding carriage of the microtome, with its long axis in the line of motion. The surface of the block is carried to and fro across the fixed knife edge, and by means of this planing action the sections are cut.

1. Length of Knife Edge and Angle of Cutting Stroke

The length of the knife is 300 mm., and a cross-section of its form is shown in fig. 2. For accurate section cutting it is necessary to reduce to a minimum the tendency of the knife edge to displace or deform the material cut. This is particularly important when the material is of the visco-elastic

FIG. 1. MODIFIED LEITZ BASE-SLEDGE MICROTOME

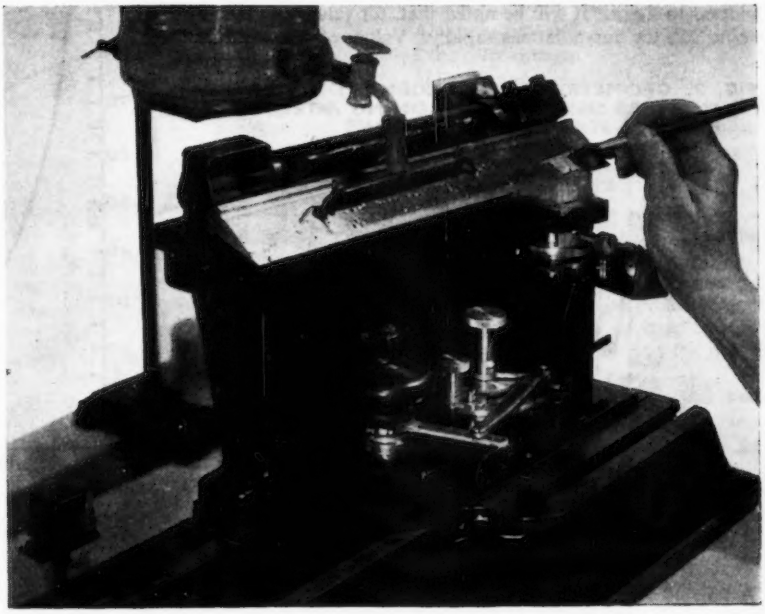
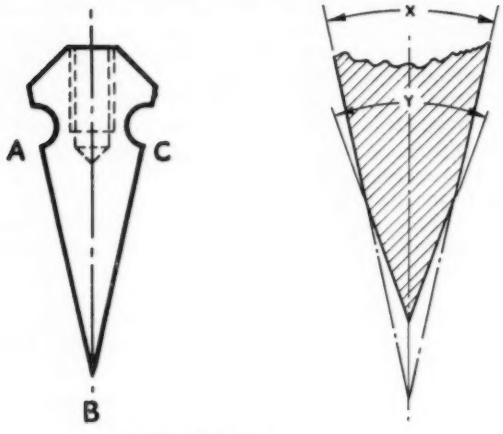


FIG. 2. CROSS-SECTION OF KNIFE

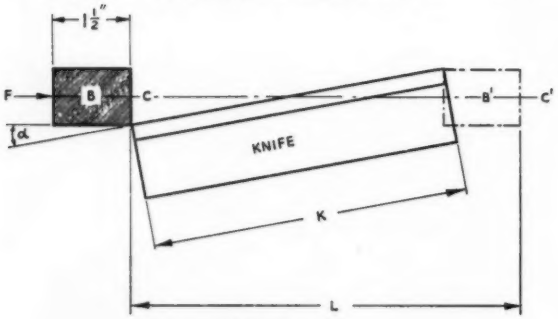


Left: actual size
Right: enlarged view of edge
Angle X: 25°
Angle Y: 40°
AB = CB = 60 mm.

consistency presented by a temporal bone embedded, in accordance with the usual practice, in celloidin.

If F is the average force applied to the carriage during the cutting of the section, and L the length of the cutting stroke, then F × L = work done. F is also the deforming force acting upon the block, and can be reduced if L is increased by an oblique setting of the knife. In fig. 3 is shown the geometrical arrangement of the equipment, as used for sectioning a temporal bone (B) of standard dimensions. Variations of F with varying values of K and α for a block of standard dimensions have been calculated and are shown plotted in fig. 4. It will be noted that, for values of α below some 20°, the curve flattens rapidly. Values of F below this

FIG. 3. GEOMETRICAL ARRANGEMENT OF EQUIPMENT, AS USED FOR SECTIONING A TEMPORAL BONE OF STANDARD DIMENSIONS



B: temporal bone
F: average force applied to carriage
L: length of cutting stroke
α: angle of cutting stroke
K: length of knife
C-C¹: line of cutting stroke

level would clearly involve very high values of K, i.e., knife edges of inconvenient length.

The relationship, experimentally determined, between α and difficulty of cutting may be stated empirically as follows: section cutting is impossible with α at 90°. There is noticeable difficulty at 50° and none at 30°.

Actual working conditions are represented by the point + upon the curve shown in fig. 4. In practice some 220 mm. of the knife edge are used. It will be noted that the position of + denotes an ample reserve of efficiency.

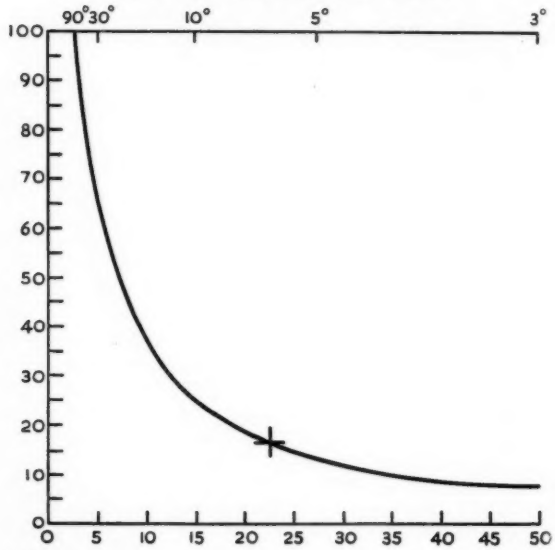
2. The Angle of the Cutting Stroke (α)

Referring again to the cross-section of the knife shown in fig. 2, it will be seen that upon the angle X (ABC) is superimposed a cutting angle Y. If the angle Y is neglected and the angle X be taken as the cutting angle, then its effective value, as presented to the plane of section, varies with the angle α of the cutting stroke. This is shown diagrammatically in fig. 5.

Thus, if α be 90°, a section of the knife taken through the line of the cutting stroke is a true transverse section, and the effective value of X presented to the plane of section will be the actual value 25°. If α is 45°, then a section of the knife through the line of the cutting stroke will take, as shown, the form of a triangle having longer sides upon the same base AC. Thus, the effective value of the angle X presented to the plane of section will be reduced.

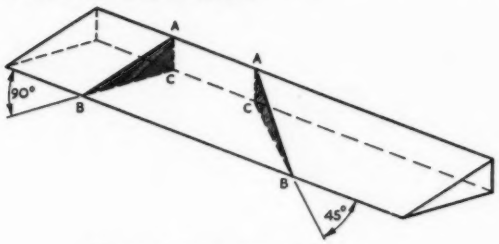
In practice an actual value of 40° for the cutting angle Y is usual, and in fig. 6 is shown the relationship of its effective value to the angle of the cutting stroke.

FIG. 4. CURVE SHOWING VARIATIONS OF F WITH VARYING VALUES OF K AND α



Abscissae:
Upper: angle of cutting stroke (α)
Lower: length of knife (K) (cm.)
Ordinates: values of force (F) required when α = 90° (%)
+ represents actual working conditions

FIG. 5. DIAGRAM SHOWING VARIATION OF EFFECTIVE CUTTING ANGLE (ABC) WITH ANGLE OF THE CUTTING STROKE



Effective cutting angle: ABC
Angle of cutting stroke (α): 90° and 45°

3. The Cutting Angle (Y)

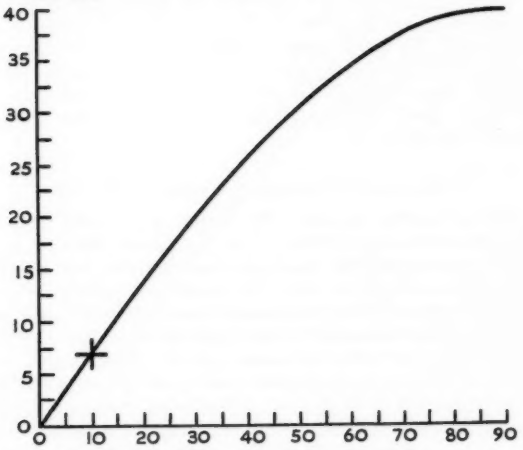
As stated, it is usual to employ an angle of cutting stroke, α, of 10° and a cutting angle, Y, of 35°–40°. This value of Y is determined by a number of factors. Of these, the behaviour of the section during the cutting stroke requires special consideration.

If the inclination of the knife to the plane of section falls below a certain value, the section, as it is cut, lies flat upon the surface of the knife, and tends to be torn away by friction during the further course of the stroke. If, however, the angle of inclination is suitably increased, then the section rolls tightly as it is cut, and is carried along without friction to the end of the stroke. This rolling of the sections is essential for efficient working, and is dependent, as in lathe operations, upon the physical consistency of the material being cut, the depth of the cut and the angle of top-rake of the cutting tool.

Using standard temporal bone blocks, and cutting at a section thickness of 25 μ., rolling is found to take place when the top-rake angle ABD (fig. 7) falls, within certain limits, below an approximate value of 135°. This value is easy to ascertain by experiment and determines the value of the cutting angle Y that has been found desirable. Thus, in order to give maximal strength to the knife edge, it is advantageous that the cutting angle Y should be as obtuse as possible. It is clear from fig. 7 that the maximum possible value of this angle DBE is 45°. Of this, 5°–10° must be allowed for clearance between the surface of the block and the metal behind the cutting edge of the knife, and the remaining 35°–40° are, therefore, available for the cutting angle Y.

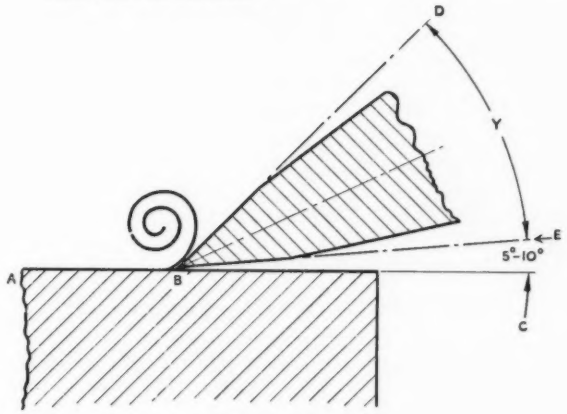
It should be noted that a factor in the high value of this angle is the obliquity of the angle α of the cutting stroke. Thus it will be seen from fig. 6 that, under the working conditions used with α = 10° and an actual value of cutting angle of 40°, the effective cutting angle presented to the plane of section is 7.25°. With a value for α, however, of 90° the same effective cutting angle, 7.25°, would necessitate an actual angle of 7.25° with a corresponding reduction of mechanical strength.

FIG. 6. CURVE SHOWING RELATIONSHIP OF EFFECTIVE CUTTING ANGLE TO ANGLE OF CUTTING STROKE



Abscissae: angle of cutting stroke (α) (deg.)
Ordinates: effective value of cutting angle (Y) (deg.)
Actual value of cutting angle (Y) = 40°
+ represents actual working point

FIG. 7. DIAGRAM SHOWING RELATIONSHIP BETWEEN TOP-RAKE ANGLE, CUTTING ANGLE AND CLEARANCE ANGLE



ABD: top-rake angle
DBE: cutting angle
EBC: clearance angle

4. Sharpening

A geometrically perfect knife edge is defined as the line of junction of two perfect plane surfaces. It follows that a satisfactory sharpening technique should be one capable of yielding perfect plane surfaces. It must be added that the size of the surfaces so produced should be commensurate with the length of the knife employed, and that the process should be speedy and easy to regulate.

Lapping on suitably charged cast-iron surface plates is well established as the best method of producing high quality plane surfaces on steel, and has been widely used and perfected by the National Physical Laboratory, Teddington, in the manufacture of high precision steel gauges. The precision of the method is such that the accuracy of the finished surfaces can be checked for flatness by interferometric methods, and lapping has accordingly been used for sharpening the microtome knives.

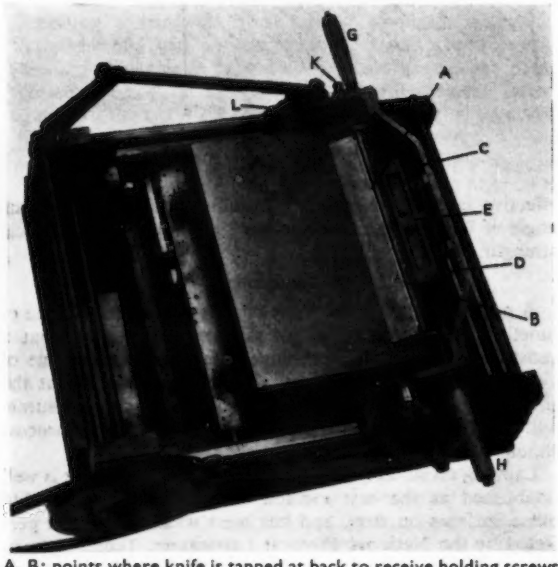
The microtome knife holder carries two V sections, which engage the back edge of the knife. The centres of these V sections are positioned symmetrically with respect to the centre of the knife, being separated by a distance of 0.55 K, where K is the length of the knife. When a bar of uniform section is supported at these points, its deformation under its own weight is reduced to a minimum. A similar arrangement for holding the knife is used in the knife-sharpening equipment, and this is shown in fig. 8.

The back of the knife is tapped at A and B and is drawn back by screws, C, D, into the V sections of the holder, and is there held securely without strain. The knife holder itself pivots about the axis E, and the knife edge is thus free to align itself upon the lapping plate. The knife edge is pushed to and fro across the plate, by means of a simple reciprocating motion at a rate of 10 cyc./min. At the same time a side-to-side motion is imparted to the plate by a crank driven from the main shaft through spiral gearing. The relative speeds of knife and plate are so adjusted that the pathway of the former upon the latter is non-repetitive. Knife and knife holder are carried upon a heavy cross member, hinged at its extremities. Reversal of the knife upon the plate is effected by turning over this cross member. During this movement, both cross member and knife are controlled by the handles G and H of the forked members which engage the extremities of the knife.

Preparation of the Lapping Plate

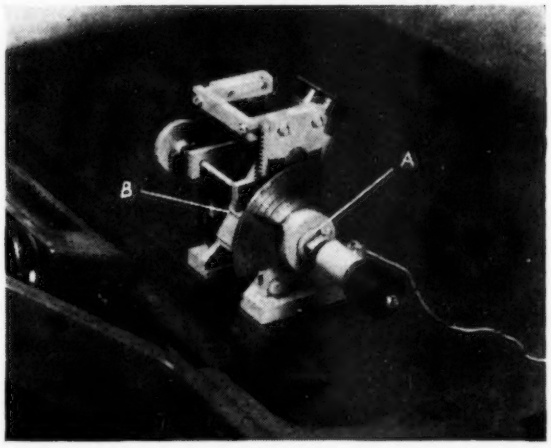
Three plates are kept in service, and their flatness is ensured by the usual technique of lapping them against each other with loose abrasive. The plates are charged with a very fine grain abrasive (800 Aloxite). This is rubbed in with a small cast-iron plate with a chequered surface.

FIG. 8. KNIFE-SHARPENING EQUIPMENT



A, B: points where knife is tapped at back to receive holding screws C, D
E: axis
G, H: handles of forked members which engage extremities of knife
K: screw-controlled distance piece
L: platform

FIG. 9. OPTICAL GONIOMETER



A: illuminated slit
B: perforated mirror

5. Control of the Cutting Angle (Y)

For this purpose, use is made of the optical goniometer shown in fig. 9. This consists of a small microscope mounted upon a flat base, for viewing the upper facet of the cutting angle as the knife edge lies in position upon the lapping plate. An image of an illuminated slit at A is reflected downwards upon the facet from a perforated mirror at B. When the barrel of the microscope is adjusted to lie at right angles to the facet, the image of the slit is reflected back along the barrel and is aligned against a cross wire in the eyepiece. The angle of inclination of the barrel can be read upon the scale with an accuracy of $\pm$ 0.25°.

By means of this instrument it is possible to read the angle of the cutting edge being applied by the sharpening procedure. This angle can then be adjusted by means of the screw-controlled distance piece K (fig. 8), which rests upon the platform L and so regulates the elevation of the cross member.

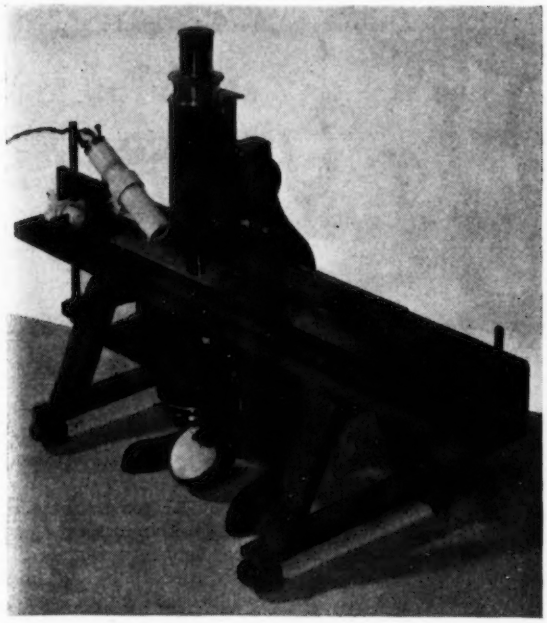
The goniometer can also be positioned firmly upon the surface of a temporal bone block while this is being cut. In this way, direct readings of the top-rake angle can be made with the knife in use.

6. Microscopic Examination of the Knife Edge

This is needed to assess the quality of the edge and the breadth of the edge facets. It is carried out with the knife laid upon a travelling cradle, as shown in fig. 10. By this means it is possible to carry out a rapid examination of the edge over its entire length at a magnification of $\times$ 60. Oblique top illumination is used for examination of the surface structure of the upper facet of the cutting angle. In addition, a low-power condenser is used for the viewing of the edge profile.

As stated, the most suitable values for the cutting angle are found to lie within the range 35°–40°. For the initial sharpening the equipment is adjusted to give an edge-angle of 35°. With repeated sharpening, the edge-angle facets become progressively wider, and the sharpening time may then become inconveniently prolonged. When the facet is shown by the inspection microscope to have attained a

FIG. 10. TRAVELLING CRADLE USED FOR MICROSCOPIC EXAMINATION OF KNIFE EDGE



breadth of 1 mm., the sharpening angle is increased by 0.5°, and a fresh edge is superimposed upon its predecessor. Its facet breadth, at first very narrow, increases with sharpening. When it has attained a breadth of 1 mm., the edge-angle is again increased by 0.5°, and so the process is continued until an edge-angle of 40° has been attained. At this point the sharpening angle is again adjusted to 35°, and the cutting angle is restored to this value by lapping with loose abrasive upon the plate. Upon the basis of this edge-angle, a fresh series of narrower edges of increasing angle is built up as before, up to a maximum value of 40°.

In fig. 11 is shown a knife edge as viewed through the edge inspection microscope. The cutting angle is 35°, and the facet breadth has reached a value approaching 1 mm. Upon this is being superimposed a fresh and narrow edge at 36°.

B. METALLURGICAL CHARACTERISTICS OF KNIVES FOR TEMPORAL BONE MICROTOMY

Our early experiences were obtained with knives of carbon steel or of various ferrous alloys, with most of which it was possible to obtain edges of very good microscopical appearance by means of our standardized sharpening procedure. In use, however, these deteriorated very rapidly and the number of serial sections obtainable seldom exceeded and often fell far short of 100.

There seemed good reason to believe that this rapid deterioration with use was due not so much to mechanical wear and tear as to corrosion by salts and other chemical constituents of the tissues, acting in association with the alcohol and water mixture used for wetting the block and the knife edge during the cutting procedure. Experiments were accordingly made with stainless steel, and the first of the knives constructed of this material by the Metrology Department of the National Physical Laboratory gave results of considerable promise. Although the microscopical appearance of its edge was obviously inferior to that obtained with our best carbon and ferrous alloy knives, it was nevertheless adequate in use for satisfactory section cutting, and series of 200 or more were regularly obtained.

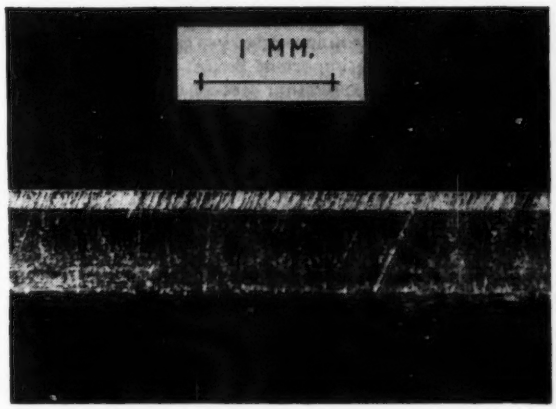
Other knives of stainless steel were accordingly constructed and, although every care was taken to control their metallurgical characteristics, the microscopical appearances of their edges presented obvious defects, and in use no satisfactory sections could be obtained. Systematic metallurgical examinations of several of these unsatisfactory knives were made in the Metallurgical Division of the National Physical Laboratory, and a number of edge defects were revealed. These were considered to account in large measure for the unsatisfactory behaviour of the knives, and were further in good accord with the results of observations of the edges carried out during the sharpening process. These revealed that, although particular identified defects were rapidly eliminated by sharpening, others as rapidly made their appearance and were due, in all probability, to minute fractures arising from faults of micro-structure. This behaviour was fully discussed with the late Mr J. E. Sears and others, and general agreement was reached that it was attributable to certain properties inherent in this type of steel.

From this we were led to abandon its use and to investigate instead certain other materials with corrosion-resisting qualities approximating to those of stainless steel, yet free from the peculiarities which rendered so difficult the satisfactory control during manufacture of its edge-taking properties. The use of one such material, Deloro Stellite alloy, for corrosion-resisting cutting edges is well established, and its development for the edges of our microtome knives was accordingly undertaken with very satisfactory results.

Stellite is a non-ferrous alloy, and is available in a number of grades of which the hardness and composition data are tabulated here:

Grade	*Cobalt* (%)	*Chromium* (%)	*Tungsten* (%)	*Carbon* (%)	*Hardness* (Vickers diamond numbers)
1	50	33	13	2.5	600
12	59	29	9	1.8	500
6	66	26	5	1.0	400

FIG. 11. KNIFE EDGE VIEWED THROUGH EDGE INSPECTION MICROSCOPE



The following are the characteristics of Stellite which prompted its use for the edges of knives used for temporal bone microtomy:

i. *Ease of application.* As in the case of other cutting tools, Stellite is deposited by welding to form the cutting edges of microtome blades of carbon or stainless steel.

ii. *Edge-taking capacity.* Experience with other edge tools made it likely that Stellite edges could be sharpened up to the standards required for temporal bone microtomy.

iii. *Resistance to corrosion.* The following data give loss in weight in mg./dm.²/day in aerated solution at room temperature:

	Sulphuric acid (10%)	Hydrochloric acid (2%)	Acetic acid (20%)
Stellite 1	0.0	2.2	3.9
,, 12	7.0	152.0	7.3
,, 6	1.9	406.0	0.6
Stainless steel (19% Cr; 10% Ni)	2.7	146.0	1.3

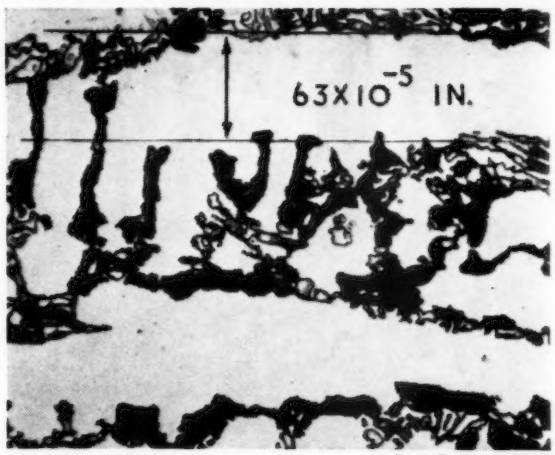
The corresponding figures for carbon steel are, of course, much higher.

iv. *Resistance of the temper of the edge to heating during re-grinding.*

v. *Ease of control of hardness and micro-structure during the manufacture.* The micro-structure of the knife edge is intimately related to its cutting qualities and will be considered in some detail. In fig. 12 is shown the micro-structure of a specimen of Stellite, etched and viewed at a magnification of × 1,000. On cooling the metal from the liquid state, precipitation and crystal growth takes place in a dendritic pattern; the carbon-free constituents of higher melting-points precipitate first and appear in the photomicrograph as the unetched white areas. On further cooling, complex carbides of tungsten and chromium are precipitated in a eutectoid structure which appears in the photomicrograph as the dark etched areas. These carbide particles have a very high hardness, and the knife edge is considered to consist of a series of such particles embedded in softer carbon-free components. It follows that fineness and evenness of edge call for a close and even spacing of these carbide particles. This fineness of structure is measured microscopically as the average distance apart of the eutectoid areas. Thus, in fig. 12, this may be taken as the separation of the ruled lines. This amounts to 63×10^{-5} in. (16.0 μ.). In practice, a knife with this micro-structure has been found to give unsatisfactory results, and a technique has accordingly been developed whereby Stellite cutting edges having a micro-structure of greater fineness can be applied to large microtome knives in a controllable manner.

In this process, the important factor is found to be the acceleration of the precipitation process by rapid cooling of the deposited Stellite. The technique is simply explained in fig. 13. An unfinished knife blade is prepared, and to its truncated edge is applied a bead of Stellite using welding rod and an oxy-acetylene flame. The blade is then placed in contact with the horizontal copper bar and the Stellite bead re-melted in contact with the copper to give a rapid cooling rate. The machining and grinding of the blade to its final form is then completed. In fig. 14 is shown the micro-structure of a Stellite knife edge deposited in this manner. The figure for its fineness of structure is, as shown in this photomicrograph, 43×10^{-5} in. (10.9 μ.). These measurements of micro-structure can be made upon the polished and etched surface of the finished blade, and are found to give a reliable indication of its cutting efficiency. Upon the basis of experience, it has, in this way, been possible to set a figure of 45×10^{-5} in. (11.4 μ.) as the upper limit for acceptance.

FIG. 12. MICRO-STRUCTURE OF SPECIMEN OF STELLITE WITH A FINENESS OF STRUCTURE OF 16.0 μ.



(magnification × 1,000)

Results. 450–550 sections normally need to be cut from temporal bone blocks. With Stellite-edged knives such a series can be cut without interruption for sharpening in a single session of 2 hours.

ACKNOWLEDGEMENT

Our thanks are due to Dr H. Barrell, Superintendent, Metrology Division, and to Dr C. H. M. Jenkins, Metallurgy Division, National Physical Laboratory, for valuable advice and assistance.

C. NITROCELLULOSE : ITS MANUFACTURE, PHYSICO-CHEMICAL CHARACTERISTICS, AND USE FOR TEMPORAL BONE MICROTOMY

The successful use of nitrocellulose as an embedding medium for human temporal bone histology depends, in large measure, upon its physico-chemical characteristics. These can now be specified and controlled in the process of manufacture, and a brief presentation of the available data follows.

FIG. 13. TECHNIQUE FOR ACCELERATION OF PRECIPITATION PROCESS DURING MANUFACTURE OF STELLITE KNIFE BLADE

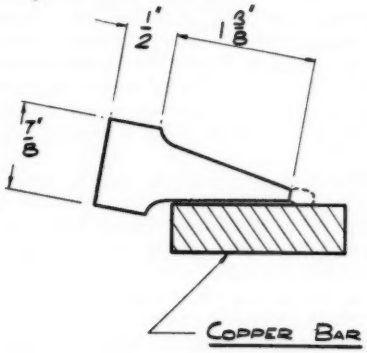
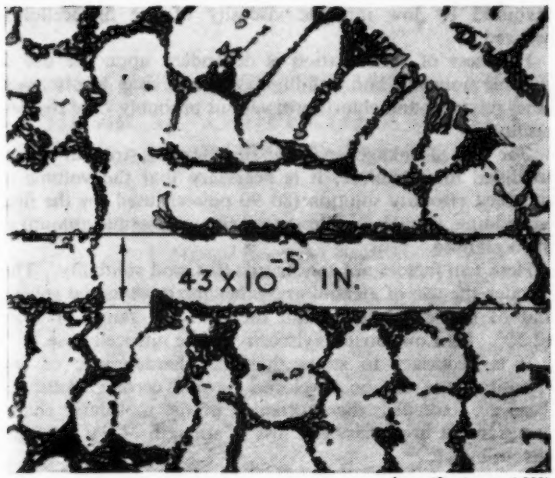


FIG. 14. MICRO-STRUCTURE OF STELLITE KNIFE EDGE WITH A FINENESS OF STRUCTURE OF 10.9 μ.



(magnification × 1,000)

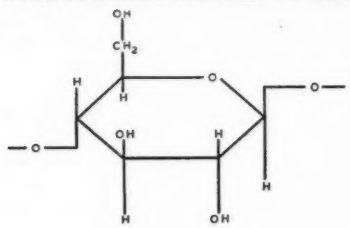
1. Manufacture of Nitrocellulose

Nitrocellulose is manufactured from cotton linters, which are the short fibres left on the cotton seed after the removal of the long fibres suitable for use in textiles. The manufacturing process consists of two stages: the first of nitration, which is related particularly to the solubility of the product; and the second of " finishing ", which is related particularly to the viscosity of its solutions. They will be separately considered.

a. Nitration

Cotton linters are nitrated by immersion in a mixture of sulphuric and nitric acid containing 12–30% water. The cellulose which forms cotton linters consists of chains of anhydroglucose units, each with three hydroxyl groups, the structural formula being shown in fig. 15. These hydroxyls are converted to nitrate during nitration to an extent depending mainly upon the amount of water present in the acid used. The extent of nitration is usually measured by the nitrogen content of the product, a nitrogen content of 14.1% corresponding to complete nitration at all three available points in each unit. The common range for industrial purposes is 10.7–12.3% nitrogen, corresponding to substitution on the average of 1.9–2.35 hydroxyls. For example, the nitrocellulose used for celluloid manufacture frequently has a nitrogen content of about 11%, whilst for lacquers a figure approaching 12% is normal. Gun-cottons are still more highly nitrated.

FIG. 15. STRUCTURAL FORMULA OF CELLULOSE



The solubility of nitrocellulose in alcohol and ether, the solvents generally used for histological work, is dependent to an important extent upon the degree of its nitration. Hence correct nitration is an important characteristic of nitrocellulose suitable for histological work, a point to which further reference will be made.

b. Finishing

The viscosity of nitrocellulose solutions is dependent upon the number of anhydroglucose units in the chains which constitute the nitrocellulose particles. This chain-length is controllable in the course of the finishing process, which consists essentially of a series of washes at various temperatures. Its first object is to ensure stability of the nitrocellulose, so that it may safely be stored, but in addition, the finishing process is adjusted so that certain desired properties, such as low viscosity in solution, are obtained.

Nitrocellulose consists of long chains, and therefore gives not a true but a colloidal solution. This means that the ultimate particles into which the nitrocellulose splits during solution, though too small to be visible to the naked eye or under the microscope, are nevertheless too large to exhibit the free diffusion and migration which is characteristic of true solutes such as salts, or simple organic compounds like sugars. During the finishing process the molecular chains are gradually broken by depolymerization into smaller fragments until the average length is that desired. The shorter the chains the less they tend to entangle when in solution, and therefore the lower the viscosity of the solution. Thus the finishing process affords a control of the viscosity of nitrocellulose in solution.

The degree of polymerization is expressed in terms of chain-length, i.e., the average number of anhydroglucose units which make up each molecular chain. This number gives a valuable indication of the viscosity of the nitrocellulose in solution.

2. Physico-Chemical Characteristics of Nitrocellulose

Measurement and specification of viscosity. The viscosity of a liquid is its resistance to shear, manifested for example by difficulty of stirring. The unit employed for its absolute measurement is the poise. This is the force in dynes required to cause a relative movement of 1 cm./sec. between two surfaces each of area 1 cm.², 1 cm. apart. Several mathematical expressions are used for the specification of viscosity. Of these the most widely used is perhaps that for intrinsic viscosity, an expression which makes possible the comparison of different varieties of nitrocellulose in respect of their viscosity under comparable conditions of concentration. In practice, however, viscosity measurements of concentrated solutions are more readily made and are of great practical value. The Ardeer[1]—or falling sphere—technique as used by Imperial Chemical Industries (I.C.I.) is perhaps the best known and most convenient of the several available.

Solution of nitrocellulose. In the process of solution the nitrocellulose particles swell and then pass into colloidal solution. There is no real upper limit of solubility, and the material continues to be miscible with the solvent at increased concentrations to give mixtures of extreme stiffness. Now the required viscosity for nitrocellulose solutions used for

[1] Ardeer, in Ayrshire, site of an explosives factory which eventually merged with the present Nobel Division of I.C.I.—ED.

histological work has a maximum figure, determined by convenience of handling. This figure may be taken as 80–90 poises, and will be referred to as the standard histological viscosity. The concentrations of different varieties of nitrocellulose which are needed to yield solutions of this standard viscosity depend upon the solvents used, and upon the degree of their nitration. Thus, alcohol alone will dissolve only nitrocellulose within the nitration range 10.7–11.0%, yielding a cloudy solution. The addition of ether, however, considerably increases its solvent power, although ether itself is a non-solvent. For reasons that will be given, high concentrations of nitrocellulose are required in solutions used for histological work. This calls for good solubility which is generally attained by the use, as solvents, of alcohol and ether mixtures. Their proportions can be varied, and optimum results are perhaps given with two parts of ether and one part of alcohol. The solvent action of alcohol and ether, as of other solvents such as acetone, is also dependent upon the degree of nitration of the nitrocellulose, and the data tabulated by Brunswig (1923) show that the optimum nitration figures lie within the range 11.8–12.2%.

Solidification of nitrocellulose. In a solution of nitrocellulose the molecules of solvent mix freely together in the so-called continuous phase. Throughout it are scattered the nitrocellulose particles in the so-called dispersed phase. In the course of solidification, or gel formation, a phase reversal occurs whereby the nitrocellulose particles link together in a lattice-work separating the solvent molecules within its meshes. Gel formation of nitrocellulose from alcohol and ether solutions may be brought about by evaporation, whereby the ether elements are chiefly eliminated. This leads to appreciable shrinkage and, in addition, the evaporation of the ether in the air tends to cause condensation of moisture which may lead to cloudiness of the nitrocellulose.

Better results are, therefore, obtained by means of chloroform vapour, exposure to which leads to replacement of the solvent ether by the non-solvent chloroform with resultant gel formation. Since, in addition, the liquid contained in the gel is not reduced, shrinkage is avoided.

3. Use of Nitrocellulose for Temporal Bone Microtomy

The foregoing data may now be considered in the light of the various practical requirements of temporal bone histology. Here good results require the fulfilment of the following conditions: (i) High speed of infiltration; (ii) evenness of solidification; (iii) low shrinkage during solidification; (iv) high mechanical strength of the solidified nitrocellulose.

These requirements can be fulfilled by means of a rational selection and use of particular solvents, types of nitrocellulose and solidifying agents.

Thus, high infiltration speed is dependent upon the use of embedding solutions of low absolute viscosity, a condition favoured by low intrinsic viscosity of the nitrocellulose selected.

Evenness of solidification is dependent upon the use of suitable solvents and solidifying agents, and alcohol and ether mixtures and chloroform vapour probably yield the best results.

For low shrinkage and high mechanical strength of the solidified nitrocellulose, it is necessary that the volume of standard viscosity solution (80–90 poises), used for the final embedding, should contain the maximum possible amount of nitrocellulose.

Here two factors are concerned: (i) Good solubility. This dictates the use of alcohol and ether mixtures as the solvent and of nitrocelluloses within the nitration range of 11.8–12.2%. (ii) Low intrinsic viscosity of the nitrocellulose.

It is necessary to stress that the characteristic of low viscosity must not be developed beyond certain limits. If these are exceeded, the shortening of the molecular chains brings about brittleness and loss of strength of the solidified nitrocellulose.

In practice a valuable guide to the selection of a nitrocellulose suitable for temporal bone histology is provided by its concentration in alcohol and ether mixture required to give a solution of standard histological viscosity. For such a determination, the falling sphere technique can be conveniently used for the adjustment of the viscosity.

Thus, for a variety of nitrocellulose found to give particularly satisfactory results (I.C.I. Type H.M. 15–20), the concentration figure was found to be 14 g./100 ml. of alcohol (1 part) and ether (2 parts).[2]

To this must be added the point already emphasized, that this concentration figure is not alone sufficient to determine the satisfactory behaviour of a given variety of nitrocellulose as an embedding medium, and additional information is also needed of its mechanical qualities in the solid state. These are expressed in terms of tensile strength (lb./in.²)[3] and of percentage elongation at the breaking-point. Complete data relating to nitrocellulose Type H.M. 15–20 are tabulated below:

Nitration	11.8–12.2%
Intrinsic viscosity	0.22 g./l.
Degree of polymerization	180
Concentration (at 80–90 poises)	14 g./100 ml. alcohol and ether mixture
Tensile strength	16,000 lb./in.²
Elongation at break	32%

It is probable that this combination is the best at present available.

[2] 14 g. total weight of nitrocellulose damped, as supplied, with industrial spirit to give 70% by weight of nitrocellulose.
[3] 1 lb. = 453.59 g.; 1 in. = 2.54 cm.—Ed.

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FORMATION, CIRCULATION AND CHEMICAL PROPERTIES OF THE LABYRINTHINE FLUIDS

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1 Normal and pathological anatomy
2 Biochemical studies
References

1. Normal and Pathological Anatomy

Until a few decades ago our views upon the nature and function of the labyrinthine fluids were based, in the main, upon the anatomical data to be found in the well-known atlases of Retzius (1881) and of Key & Retzius (1875). These showed that the perilymph spaces, connected through the cochlear aqueduct with the cerebrospinal subarachnoid cistern, are in effect a diverticulum of the latter in which is immersed the closed sac of the membranous labyrinth, derived from the invaginated ectoderm of the otic vesicle and filled with a fluid of its own, the endolymph.

The picture is incomplete, and has not escaped critical scrutiny by later investigators, some of whom (Waltner, 1948) have considered that the labyrinthine opening of the aqueduct is barred by a limiting membrane derived from the arachnoidal web which is often present at this point.

In the last twenty years the pace of progress has increased, and many additions have been made to our knowledge of the physiology and pathology of the internal ear. As a result, the attention of investigators has been particularly directed towards the endolymph system, and here the difficulties encountered have been very great.

Once again, our views of its source and circulation find their origin in morphological studies, and the histological investigations of Shambaugh (1908) provided valuable evidence of its origin as a secretion of the stria vascularis, a gland-like structure in the outer wall of the scala media of the cochlea. In 1927 the matter was carried a stage further by the publication by Guild of his well-known application of the Prussian-blue granule technique to the endolymph system of the guinea-pig, whereby he was able to adduce evidence very suggestive indeed of a current flow from the endolymph scala of the cochlea to the saccule and finally through the wall of the saccus endolymphaticus.

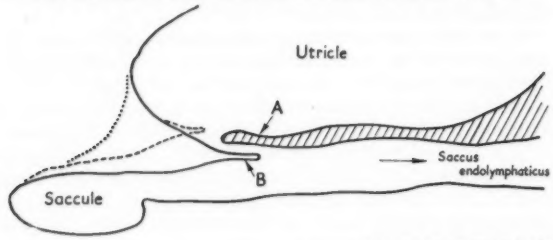
Since then, attention has been further focused upon the endolymph by the results of histological studies of the labyrinth in two well-known disorders of the inner ear: Ménière's disease and certain forms of inherited deafness in man and animals. In Ménière's disease, a disorder apparently peculiar to the human labyrinth, severe attacks of vertigo occur in association with deafness and tinnitus. Histological examination of the labyrinth shows distension of the endolymph system, chiefly affecting its cochlear portion and the saccule. Degenerative changes of the sensory cells, in particular the hair cells of Corti's organ, are also found. Degenerative changes, beginning in early life and again chiefly localized to the endolymph system of the cochlea and saccule, are also characteristic of one of the commoner forms of inherited inner-ear disease in man, the so-called cochleo-saccular degeneration of Scheibe. These parts of the membranous labyrinth are commonly found collapsed with severe degenerative changes of their contained sensory end-organs. Similar findings within the labyrinth are also known to occur in association with inherited forms of inner ear deafness observed in certain breeds of white cats, white bull terriers, and mice.

In all of these conditions the degenerative changes in the cochlear end-organs are accompanied by changes in the stria vascularis, and the parallel course of their development was demonstrated with particular clarity in the shaker-1 mouse (Grüneberg, Hallpike & Ledoux, 1940). By contrast, the fluid spaces and sense organs of the utricle and semicircular canals are comparatively little affected in any of these conditions. (Photomicrographs illustrating the pathological changes which occur in the endolymph system in Ménière's disease of the labyrinth and in the inherited deafness of the white cat and the shaker-1 mouse are shown in Plates I and II, figs. A–R.) The findings have been taken to provide strong circumstantial evidence that the endolymph is secreted by the stria vascularis, and is further responsible for the maintenance of the metabolic processes of Corti's organ.

The finding of normal conditions in the utricle and canal system, coincident with destruction of the cochlear part of the endolymph system, is difficult to explain and has been held to suggest (Seymour, 1954) that the fluid system of this part of the membranous labyrinth can be maintained from some other source, e.g., the saccus endolymphaticus. In this connexion Seymour finds an interesting functional application for an anatomical feature of the membranous labyrinth to which attention was first directed by Bast (1928), the utriculo-endolymphatic valve. According to this author, this " valve ", the connective-tissue fold shown at A in fig. 1, allows fluid to flow from the saccus endolymphaticus into the utricle, but prevents its return. Seymour's histological observations upon the saccus itself also led him to conclude that it has an important secretory function. Its fluid contents, formed in situ, would thus be able to pass into the utricle and semicircular canals, and maintain their function without reference to the cochleo-saccular portion of the membranous labyrinth.

It is, however, noteworthy that the extensive histological studies by Secretan (1944), carried out in Nager's laboratory at Zurich, led him to conclude that the histological features of the saccus described by Seymour should rather be interpreted as evidence of its absorptive function.

FIG. 1. THE UTRICULO-ENDOLYMPHATIC VALVE OF BAST, AND THE UTRICULO-SACCULAR FOLD



Redrawn from Secretan (1944)

A : utriculo-endolymphatic valve of Bast

B : utriculo-saccular fold. It may occupy the positions shown by the interrupted lines, permitting direct communication between the saccule, utricle and saccus endolymphaticus

In addition, Secretan carried out a careful examination of the anatomy of the utriculo-endolymphatic valve and found therein good ground for doubting Bast's interpretation of its function. Secretan, though he does not deny the existence of the "valve" as described by Bast, points out that its individual variations may be very considerable. In addition, he stresses the existence in this region of another connective-tissue fold which is shown at B in fig. 1. This fold, which he terms the utriculo-saccular fold, is very much lighter in structure than the formation described by Bast, and is for this reason much more likely than the latter to act as a mobile valvular element. In this event there would be no anatomical basis for a one-way movement of fluid from the saccus to the utricle, as suggested by Seymour.

2. Biochemical Studies

The controversial nature of the conclusions which can be drawn from this and other morphological studies has long made evident the need for more penetrating methods of inquiry, and recent biochemical studies of the labyrinth fluids have added much to our knowledge of their nature and function. The work of Kaieda (1930) and of Jensen & Vilstrup (1954) upon the labyrinthine fluids of the elasmobranchs is well known. Here the labyrinths are accessible, and quantities of up to 0.5 ml. are obtained. It is, however, difficult in view of the fact that post-mortem material was used, and in view, too, of the wide difference between the species, to relate their findings to conditions in the mammalian labyrinth.

In the mammal nearly all of the investigations reported have been carried out upon the cat and the guinea-pig. In both species perilymph can readily be obtained by puncture of the round-window membrane. Much greater technical difficulties have been encountered, however, in obtaining uncontaminated samples of endolymph. In addition, the samples so obtained are extremely small, and the consequent difficulties of their chemical examination are very great.

Measurements of the osmotic pressure of the labyrinthine fluids of the cat were reported in 1940 by Aldred, Hallpike & Ledoux, and this was followed by the valuable studies of Ledoux (1941, 1943, 1950) of the physical and chemical properties of these fluids. These results are summarized in Table I.

More recently, an important contribution has been made by Smith, Lowry & Wu, whose publication (1954) provides a detailed account, the first of its kind available, of measurements, carried out by means of modern microchemical techniques, of the electrolyte contents of the labyrinthine fluids of the guinea-pig (see Table I).

A systematic study of the composition of the labyrinthine fluids in these two species has recently been initiated by the Otological Research Unit and the Unit for Research in Cell Metabolism of the Medical Research Council with a view to developing new ultra-microanalytical methods for the analysis of these very small samples, since most of the existing micromethods are not sensitive enough. A brief preliminary account will be given of the methods used and the results so far obtained, together with a review of the data already available.

Methods. The collection of the fluid samples was carried out at the Otological Research Unit of the Medical Research Council at the National Hospital, Queen Square. General anaesthesia with ether and oxygen was used. The perilymph samples in the cat were collected in the usual way by puncture of the round-window membrane. In the guinea-pig, however, it was generally found more convenient to collect them from the perilymph space of the vestibule after removal of the stapes preparatory to collection of the utricular endolymph as shown in Plate II, fig. T. Endolymph samples were collected, either from the cochlea by means of the technique described by Aldred *et al.* (1940) as illustrated in Plate II, fig. S, or from the utricle of the guinea-pig by the method of Smith *et al.* (1954) as illustrated in Plate II, fig. T.

It was found possible to obtain perilymph in amounts of 3.0 µl. or more from either animal. With the endolymph, however, the amounts obtained were generally of the order of 0.1 to 0.5 µl. The special ultra-micropipettes used were manufactured from boro-silicate glass.

All samples shown by microscopic examination to contain more than a very few red blood cells were rejected. Each sample was drawn by suction well away from the tip of the pipette which was then sealed at both ends over a flame, placed in a protective sheath and transported in a vacuum flask packed with solid CO_2 to the Department of Biochemistry, University of Oxford, where the chemical analyses were carried out.

Sodium and potassium were measured by flame photometry. The flame photometer was a Beckman DU model fitted with a photomultiplier attachment. The samples were transferred from the collecting pipette via a silicone-treated microscope slide to a measuring ultra-micropipette in which 0.2 µl. occupied about 20 mm. of capillary length. The length of column occupied by the sample was measured, and blown into 1 ml. of 40% pure acetone. Standard sodium and potassium solutions were drawn into the same length and treated in the same way. Sample solutions and standards were compared, using an oxygen-hydrogen flame. The amounts measured were 120 µmg. $\pm$ 3% sodium, and 40 µmg. $\pm$ 6% potassium in the case of the smaller samples. Chloride was measured by modifying an existing potentiometric method. Silver nitrate was titrated against the sample with an ultra-microburette.

Total nitrogen and non-protein nitrogen were estimated by a colorimetric method (Exley, 1956). The principle was to digest the organic nitrogen with sulphuric acid containing selenium catalyst in a modified (Bruel, Holter, Linderstrøm-Lang & Rozits, 1947) ultra-micro-Kjeldahl digestion apparatus, and to add sodium phenate and potassium hypochlorite directly to the neutralized digest. The absorption of the blue

LABYRINTHINE FLUIDS

(FIGS. A—T)

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FIGS. A–D. Photomicrographs illustrating pathological changes which occur in the organ of Corti in inherited deafness of the shaker-1 mouse

(magnification × 180)

FIG. A. Normal mouse (50 days)

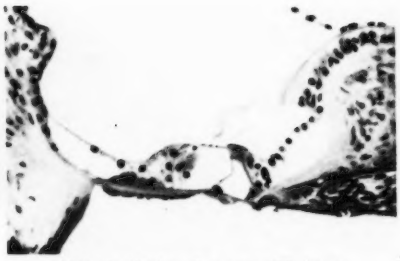

FIG. B. Shaker-1 mouse (38 days)

FIG. C. Shaker-1 mouse (117 days)

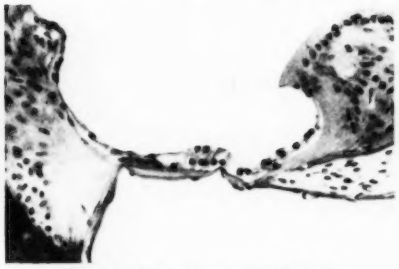

FIG. D. Shaker-1 mouse (216 days)

FIGS. E–H. Photomicrographs illustrating pathological changes which occur in the stria vascularis in inherited deafness in the shaker-1 mouse

(magnification × 180)

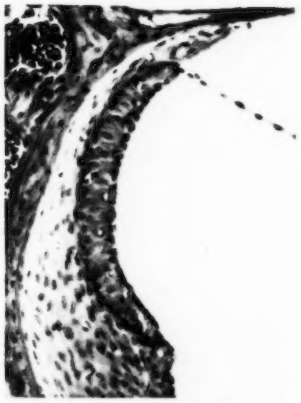

FIG. E. Normal mouse (50 days)

FIG. F. Shaker-1 mouse (38 days)

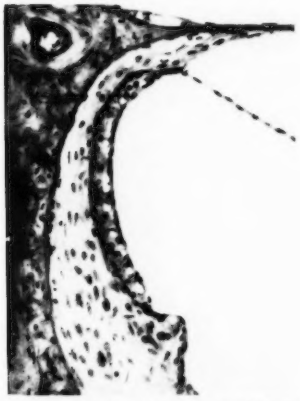

FIG. G. Shaker-1 mouse (117 days)

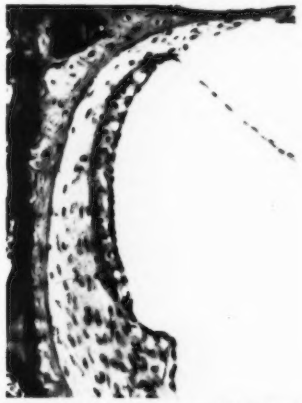

FIG. H. Shaker-1 mouse (216 days)

FIGS. J–P. Photomicrographs illustrating changes which occur in the endolymph system in Ménière's disease

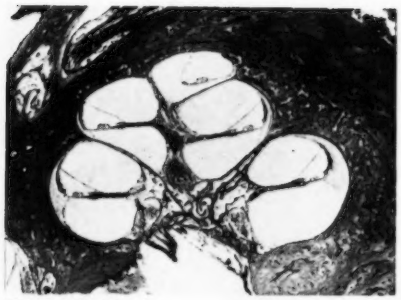

FIG. J. Cochlea in normal subject (magnification × 8)

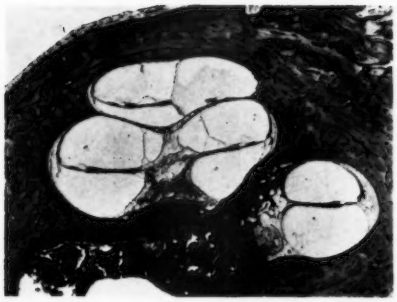

FIG. K. Cochlea in subject with Ménière's disease (magnification × 8)

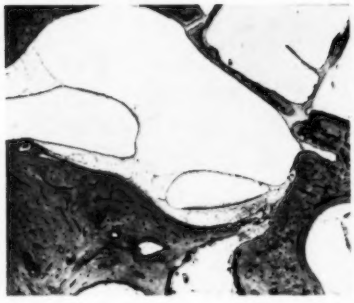

FIG. L. Saccule in normal subject (magnification × 8)

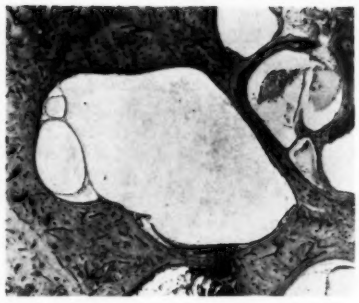

FIG. M. Saccule in subject with Ménière's disease (magnification × 8)

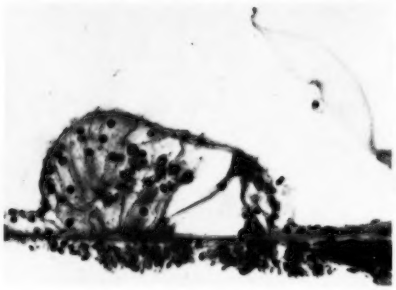

FIG. N. Organ of Corti in normal subject (magnification × 180)

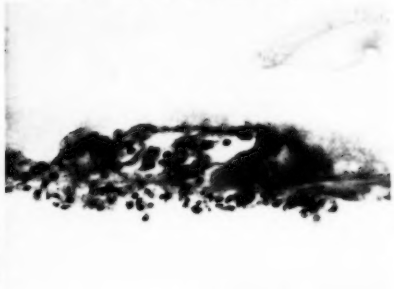

FIG. P. Organ of Corti in subject with Ménière's disease (magnification × 180)

FIGS. Q, R. Photomicrographs illustrating changes which occur in the organ of Corti and the stria vascularis in inherited deafness in the white cat (magnification × 58)

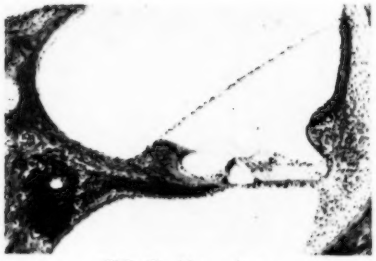

FIG. Q. Normal cat

FIG. R. Inherited deafness in white cat

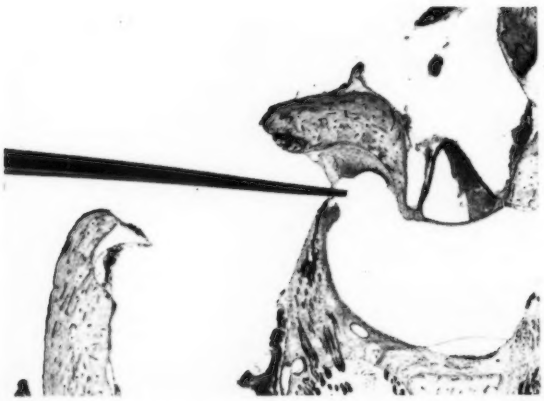

FIG. S

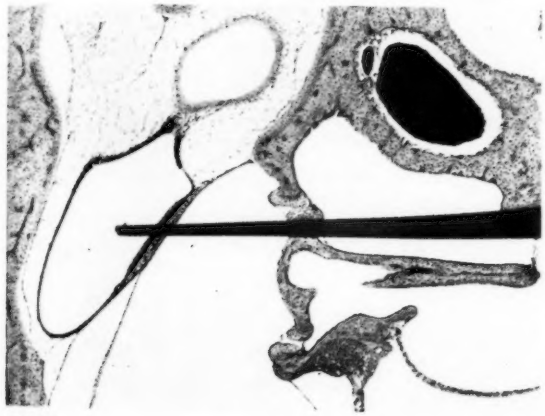

FIG. T

FIGS. S, T. Collection of endolymph from cochlea of cat (fig. S), and from utricle of guinea-pig (fig. T) (magnification × 30)

TABLE I. ANALYSIS OF LABYRINTHINE FLUIDS IN THE CAT AND GUINEA-PIG: RESULTS OF PREVIOUS INVESTIGATIONS

Animal	Properties and constituents	Spinal fluid	Perilymph	Endolymph	Reference
Cat	Osmotic pressure (g. NaCl/100 g. H_2O)	1.017	1.046	1.058	Aldred, Hallpike & Ledoux (1940)
	Index of refraction ($n_D^{22°C.}$)	1.33435	1.33495	1.33455	Ledoux (1941)
	pH	7.45	7.87	7.82	Ledoux (1943)
	Chloride (m-equiv./l.)	152	158	151	Ledoux (1950)
	CO_2 (vol./100 ml. s.t.p.)	43.2	32.0	31.5	
	Protein (mg./100 g.)	31	268	118	
Guinea-pig	Sodium (m-equiv./l.)	152	150	16	Smith, Lowry & Wu (1954)
	Potassium (m-equiv./l.)	4.2	4.8	144	
	Chloride (m-equiv./l.)	122	121.5	107	

indophenol colour produced was measured by a Beckman spectrophotometer, using special capillary cuvettes made from Fluon. These cuvettes held 100 µl. and possessed a 5 cm. light path. This method made it possible to estimate 20–100 µmg. ± 6% organic nitrogen in 0.1–0.5 µl. samples. The volume of these samples was measured to the nearest 0.001 µl. by a flame photometric method. Non-protein nitrogen was estimated in samples after precipitating the protein with the Folin-Wu reagent in ultra-microcentrifuge tubes.

Before describing the results, the findings of other workers will be reviewed. These are shown in Table I.

All of the investigators named collected the perilymph specimens by puncture of the round-window membrane. Endolymph specimens were collected in the cat from the scala media, as shown in Plate II, fig. S, and in the guinea-pig from the utricle as shown in Plate II, fig. T.

Two points of evident significance emerge from these results and call for comment and comparison with our own findings: firstly, Ledoux's (1943) finding in the cat of a protein content higher in both endolymph and perilymph than in the cerebrospinal fluid (c.s.f.); secondly, the remarkable finding of Smith *et al.* (1954) that, whereas the concentration of sodium and potassium in the perilymph of the guinea-pig (150 and 4.8 m-equiv./l. respectively) differed little from those in the c.s.f., these values were substantially reversed in the endolymph.

In addition to these two points, small but significant differences of osmotic pressure and refractive index of the various fluids were recorded and appear in Table I. Our own results are shown in Tables II and III, and summarize the results obtained in a total of over 250 analyses. The bracketed figure appended to each entry represents the total number of observations upon which it is based. It will be seen that we have confirmed the high potassium, low sodium content reported by Smith, Lowry and Wu in the endolymph of the guinea-pig, and a similar trend was also found in a number of observations upon the cat.

In both animals the non-protein nitrogen content of the labyrinthine fluids did not differ from that in the c.s.f. In both animals, however, the protein content in the perilymph was a good deal higher than that in the c.s.f.

In contrast with Ledoux, however, we found no difference in the protein content between the utricular endolymph of the guinea-pig and its c.s.f.

Discussion

It seems not unlikely that the biochemical data presented, though far from complete, will be found to possess a highly significant relationship to many of the problems connected with the nature and function of the labyrinthine fluids. Thus, the fact that the protein concentration of the perilymph is

TABLE II. ANALYSIS OF LABYRINTHINE FLUIDS IN THE GUINEA-PIG: RESULTS OF PRESENT INVESTIGATIONS

Constituent	Serum	Spinal fluid	Perilymph	Utricular endolymph
Sodium (m-equiv./l.)	140 (4)	150 (12)	148 (12)	26 (8)
Potassium (m-equiv./l.)	4.5 (4)	4.0 (12)	5.0 (12)	142.0 (9)
Chloride (m-equiv./l.)	—	122 (9)	120 (9)	110 (3)
Protein (mg./100 g.)	—	20 (6)	75 (6)	25 (6)
Non-protein nitrogen (mg./100 g.)	—	21 (6)	20 (6)	21.5 (6)

TABLE III. ANALYSIS OF LABYRINTHINE FLUIDS IN THE CAT: RESULTS OF PRESENT INVESTIGATIONS

Constituent	Serum	Spinal fluid	Perilymph	Cochlear endolymph
Sodium (m-equiv./l.)	155 (12)	162 (12)	164 (12)	66 (12)*
Potassium (m-equiv./l.)	6.7 (12)	5.9 (12)	6.0 (12)	117.0 (12)*
Chloride (m-equiv./l.)	—	150 (4)	150 (4)	—
Protein (mg./100 g.)	—	25 (5)	142 (5)	—
Non-protein nitrogen (mg./100 g.)	—	20 (5)	21 (5)	—

* In many of these samples the Na^+ and K^+ concentrations suggested contamination with perilymph. Three samples, however, showed the high values for K given in the table.

higher than that of the c.s.f. has an obvious bearing upon current views on the interchange of these fluids thought to take place through the cochlear aqueduct, and might well be interpreted as confirming the existence at its cochlear extremity of a semipermeable barrier membrane which retains the larger protein molecules within the perilymph, while permitting interchange of the smaller electrolytes. On the other hand, such an explanation may not be necessary if the possibility can be accepted that the high protein content of the perilymph is a species of stagnation phenomenon akin to that which may occur in the subarachnoid space in association with localized restrictions in the circulation of the c.s.f. and with increase of its protein content. This latter situation clearly bears some resemblance to the anatomical picture of the perilymph space drawn by Key & Retzius (1875) as a narrow-necked diverticulum of the subarachnoid space.

The biochemical characteristics of the endolymph call for special comment, in particular the combination therein of high potassium and low sodium concentrations. These data appear to be unique for extracellular fluids and would seem to establish a biochemical similarity between endolymph and other intracellular fluids elsewhere in the body. Although their significance is still unexplained, they provide, nevertheless, strong support for the view that the endolymph is produced by the secretory activity of some specialized cells, though whether these are to be found in the stria vascularis, as claimed by Guild, or in the saccus endolymphaticus, cannot at present be stated. It is possible that both structures are concerned, and perhaps others, too, not yet identified.

Certain neurophysiological problems are also imported by the unusual sodium and potassium contents of the endolymph, and Smith *et al.* (1954) have commented upon the physiological difficulties of nerve-impulse transmission along the un-medullated nerve fibres which traverse the tunnel of Corti's organ apparently immersed in the endolymph. No clear solution of this difficulty can at present be given and it is possible that the fluid system within Corti's organ itself is an enclave separated from the endolymph space from which our samples have been collected, and containing fluid with biochemical properties which do not differ from those of the extracellular fluids which surround nerve fibres in other parts of the body.

ACKNOWLEDGEMENTS

The authors wish to thank Professor H. A. Krebs, F.R.S., and Dr D. E. Hughes for their encouragement and interest in this work.

Acknowledgement is also made to the Royal Society for permission to reproduce Plate I, figs. A–H.

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ELECTROPHYSIOLOGY OF THE CENTRAL AUDITORY PATHWAY

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1 Early attempts at electrical recording
2 Structural organization of the pathway
3 Response to tonal stimuli
4 Temporal relations
5 The role of inhibition
6 Summary
References

Any consideration of the part played by electrophysiological studies of the auditory pathway cannot be completely separated from the general study of the mechanism of hearing. A review of this subject cannot be undertaken in the space of a short article, and many such reviews already exist, e.g. Wever (1949) and Galambos (1954). Attention will therefore be confined to considering some of the contributions that electrical recording has made to the elucidation of the role of the pathway from inner ear to the higher centres in relation to the whole auditory process, a subject which has recently made considerable progress.

It is only in comparatively recent years that any detailed consideration has been given to the idea that the nervous pathway, as such, contributed anything beyond its obvious function as a means of conveying information from one end of the system to the other. The ever-popular resonance hypothesis, with which the name of Helmholtz is associated, assumed that a peripheral analysis of complex sounds into their component frequencies was carried out mechanically by the inner ear, and that the appropriate auditory nerve fibres "corresponding" to these frequencies were activated—the spatial pattern of activity so created being carried unchanged to the higher centres. A rival group of theories, coming under the general heading of telephone or temporal theories, demanded that a replica of the actual sound frequencies be transmitted, again substantially unchanged, to some centre capable of "appreciating" such frequencies. The problem was not capable of being settled until the advent of electrical methods of recording nerve activity made it possible to study the question experimentally.

1. Early Attempts at Electrical Recording

The earliest records of an electrical nervous response to a sound stimulus were made by Beck (1890), who led from electrodes on the cortical surface to a sensitive galvanometer and showed that potential changes were produced by loud sounds of the calibre of pistol shots. At about the same time, apparently successful attempts were made to record electrical responses from the auditory nerve under similar conditions, though in the light of later knowledge it seems probable that these were in fact cochlear responses. Although some improved results were later obtained with the string galvanometer, it was not until the development of electronic amplifying and recording apparatus in the 1920's that any detailed information about the behaviour of the auditory system became available.

Adrian & Zotterman, in 1926, recorded the electrical discharge from a single end-organ (a stretch receptor) and found rates of discharge not exceeding 200/sec. This lent weight to the idea, derived earlier from studies of refractory period in motor nerves, that a single fibre could not achieve firing rates high enough to fulfil the requirements of a simple telephone theory unless the auditory nerve fibres had quite unique properties.

The first usefully detailed records from the inner ear were obtained by Wever & Bray (1930), who, in a famous series of experiments, showed that an electrical replica of a stimulating tone could be obtained from suitably placed electrodes, over a very wide range of frequency. These records, which appeared to support a telephone hypothesis, subsequently turned out to be a mixture of nerve action potentials, and voltages produced within the ear itself—the cochlear microphonic potentials. The action potentials can be recorded uncontaminated, by means of a concentric electrode suitably placed on the auditory nerve (Derbyshire & Davis, 1935). It was found by this method that synchronism of the action potentials with the stimulus waveform fails progressively at 3,000–4,000 cyc./sec. Galambos & Davis, in 1943, recording activity from single cells at the root of the auditory nerve, showed (i) that synchrony of the impulses with the stimulating sinusoid was present at low frequencies but was imperfect, at this level, even at 1,000 cyc./sec.; (ii) that the maximum adapted rate was only about 200/sec.; and (iii) that while each unit had a corresponding characteristic frequency to which it was most readily responsive, at intensity levels of 20–30 db. above threshold a given unit might respond to any frequency within a range of about two octaves.

At this stage sufficient evidence had accumulated to show that while both the resonance and telephone hypotheses had some elements of truth, neither, in its pure form, was capable of meeting the experimental facts. It is from this point that modern ideas of the role of the nervous pathway as an active participant in the hearing mechanism date.

2. Structural Organization of the Pathway

The main pathways involved in the auditory nervous system have been worked out by the standard anatomical techniques, and electrophysiology has been able to do little more, so far, than confirm the existence of activity in all these pathways in response to sound stimuli (Kemp, Coppée & Robinson, 1937). The precise way in which the various parts of the auditory pathway are interconnected is still rather poorly understood, and electrical recording techniques could undoubtedly make a considerable contribution if the problem were tackled systematically. The main contribution so far has been the demonstration that the detailed orderliness of the projection of the cochlea on the cochlear nucleus is maintained right up to the cortical level. Galambos, Rose & Hughes (1951) showed that there was a progressive and orderly change in frequency sensitivity of the units as a microelectrode was passed through one particular region of the cochlear nucleus. The experiments of Woolsey & Walzl (1942) in the cat, and of Tunturi (1944) in the dog, showed

that stimulation of a point on the spiral lamina of the cochlea produced activation of a corresponding point on the cortex, and that the two sets of points were related in an orderly manner. From these two series of findings it would seem reasonable to infer that there is a spatially orderly arrangement of connexions throughout the pathway. The demonstration of this general orderliness is in no way at variance with the probability that there is considerable randomness of connexion in detail, or with the demonstrable occurrence of considerable (but still orderly) lateral overlap between adjacent channels.

3. Response to Tonal Stimuli

The ear has a remarkable facility for assessing pitch, and, since the time of Helmholtz and before, there has been a tendency to concentrate on this aspect of hearing. However, the ability really only manifests itself under rather restricted conditions. For stimuli lasting 10 msec. or less, the pitch judgement is poor, and below about 5 msec. it is absent. This correlates with the observed electric response, where at a cortical level the response to any short-lasting tonal stimulus cannot be distinguished from that to a click. Again, it is well known that the apparent pitch of a pure tone varies with its intensity (Weber, 1828; Stevens, 1935), yet this effect is not normally observed with the complex tone of a musical instrument. The relative proportion of overtones in the same note played by different musical instruments is very different, and differences in tone quality have been said to depend on an analysis of this harmonic content. Yet, provided the onset of the note is not heard, it is quite difficult to distinguish between the same note played on instruments as different as the trumpet and the flute. The peculiar effect produced by " reversing in time " each note in a sequence played on the piano is quite remarkable when first heard. Thus, although the frequency discrimination of the ear can be extremely good under certain well-defined conditions, it is not so if other factors have to be simultaneously considered.

Most, if not all, frequency studies have been made with single-tone stimuli, or at most with two independent tones, mainly owing to the simplicity and ease of handling of this stimulus. Many of these studies have been made with steady-state stimuli, but a number have relied on " onset tones " or " tone pips ". While these latter methods may be useful at low neural levels they are almost certainly invalid at the cortex.

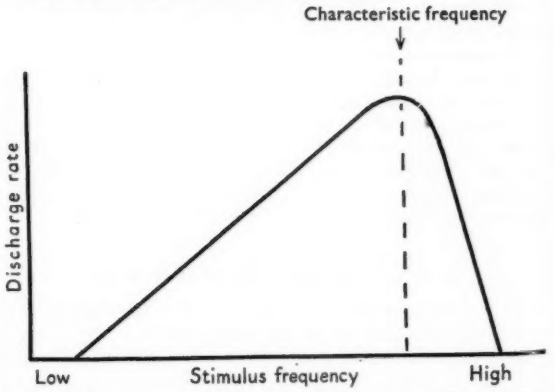
Information about single-unit activity in the auditory nerve itself has been lacking until comparatively recently. However, in 1954, Tasaki succeeded in obtaining records for brief periods from such units in the guinea-pig. Although in most cases he was not able to hold a unit for more than about 10 seconds, in a few cases recording was continued for a long enough period to enable a rapid survey of the threshold/frequency relation to be made with tone pips. The curves so obtained correspond rather well with the studies of the mechanical activation of the basilar membrane made by Békésy (1943), and by Tasaki, Davis & Legouix (1952); that is to say, a threshold response curve was obtained which cut off steeply on the high-frequency side, but much less steeply on the low-frequency side. Because of the use of short stimuli, it is not possible to assess the relation between stimulus intensity and discharge rate, except in so far as it is clear that the relation is a direct one.

The earliest point on the pathway for which detailed information is available is at the root of the auditory nerve. Galambos & Davis (1943) recorded activity from structures in this region, which they subsequently thought to be aberrant cells of the cochlear nucleus. These cells have threshold response curves of the same general form as that found by Tasaki, but with a rather more steep rise on the low-frequency side; moreover, the units responding at high frequencies have a narrower response band than those responding at low frequencies. The discharge rate of these units varies with intensity in the usual sigmoid manner associated with peripheral sensory elements, and reaches a maximum adapted rate of about 200/sec. at intensity levels around 30 db. above threshold.

Combining these two sets of results, we can obtain a relation between the stimulus frequency and the response of a single unit, such as is shown diagrammatically in fig. 1. We may characterize such a unit by the frequency to which it responds optimally (its " characteristic frequency "), and since there is no reason to think that units with adjacent characteristic frequencies do not have essentially similar response curves, it is possible to construct a diagram showing the relative activity in the whole array of afferents to the cochlear nucleus when the ear is stimulated by a given tone at a given intensity (Allanson & Whitfield, 1956) (fig. 2a). The fibre or group of fibres being stimulated at their characteristic frequency, discharge at the highest rate, while those whose characteristic frequencies differ from the stimulus frequency respond proportionately less strongly. Owing to the asymmetry mentioned in the preceding paragraph, more fibres with characteristic frequencies above the optimal than below it are found responding.

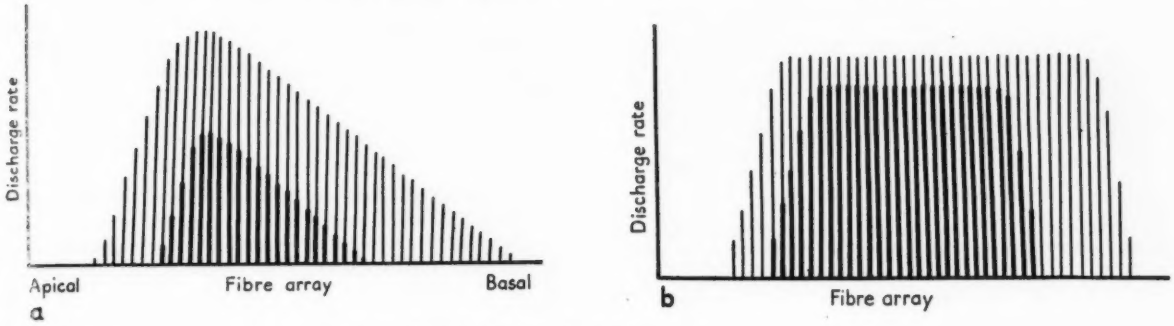
The threshold/frequency response curves appear to remain in the same form at all levels right up to the cortex (Hilali & Whitfield, 1953; Gross & Thurlow, 1951; Hind, 1953). However, this is not true of some of the intensity/discharge-rate

FIG. 1. THE RELATION BETWEEN DISCHARGE RATE AND STIMULUS FREQUENCY IN A SINGLE UNIT ON THE AFFERENT SIDE OF THE COCHLEAR NUCLEUS ("AUDITORY NERVE")



Each unit has a " characteristic frequency ". For a given intensity, a sound stimulus at this frequency produces a more rapid discharge than a stimulus at any other frequency. The rate of firing declines progressively as the characteristic frequency is departed from, and does so much more rapidly for stimulus frequencies above the optimum than for those below it.

FIG. 2. DIAGRAMMATIC REPRESENTATION OF DISCHARGE PATTERN IN THE AUDITORY NERVE, AND IN THE EFFERENT PATHWAY FROM THE COCHLEAR NUCLEUS



a: pattern of activity in the auditory nerve
b: corresponding pattern in the efferent pathway (trapezoid body)

Each line represents 10–20 fibres, and its height their mean nerve impulse rate. The relative position of each line in fig. 2a represents the relative point of origin of the fibres concerned, along a portion of the basilar membrane. Thus the lines toward the left of the diagram represent fibres arising from a more apical position, and hence responding preferentially to lower frequencies. Figure 2b is arranged in the same way, the "left-hand" fibres having the lower characteristic frequencies. The thicker portions of the lines in each case indicate the response to a low-intensity stimulus, the whole lines that to a higher intensity.

relationships. Hilali & Whitfield (1953) studied the behaviour of single units on the efferent pathway from the cochlear nucleus and found not only that the maximum discharge rates were lower than those found by Galambos and Davis, but also that the rates attained were very much less dependent on intensity; thus any particular unit had a much more nearly "on-off" relationship to the stimulus. The effect of this is that, at the level studied, there is a tendency for a particular block of fibres to be strongly active, while being flanked on each side by completely inactive fibres (fig. 2b). Altering the frequency changes the position of the block, while altering the intensity merely makes it wider or narrower, without strongly influencing the rate of discharge of the central fibres of the group. It would thus seem that the frequency and intensity information is concentrated, by the action of the cochlear nucleus, into the regions of change between activity and inactivity. There does not appear to be any reduction of the number of units responding to a particular frequency and intensity as we ascend the neural pathway, although Erulkar (personal communication, 1955), working at Oxford, has found some units with quite narrow response bands in the inferior colliculus. It is conceivable, as Dr A. M. Uttley has suggested, that these are units responding to the edges between active and inactive regions, and hence signalling the position of these.

As has been previously noted, there appears to be an orderly anatomical projection of the cochlea on the auditory cortex. Experiments undertaken under various abnormal conditions indicate the existence of a tonotopic organization of the cortex. The experiments of Tunturi (1944), using onset tones in the anaesthetized animal, gave rather poorly defined and overlapping areas for different tones, while similar work with anaesthetized strychnine-treated cortex (Tunturi, 1950) gave a fairly precise location for each tone. However, since the onset period of a tone cannot be regarded as a pure tonal stimulus (if of abrupt onset, it is found to give a unit response similar to that of a click), and since quite concentrated strychnine solutions (3%) needed to be used, neither set of results can be regarded as conclusive proof of such organization. Bremer (1943), who recorded the electrical activity of the auditory cortex in the unanaesthetized *encéphale isolé* in cats, was unable to find any tonotopic distribution of activity, although he noted that a region "fatigued" to one tone would respond to another adjacent tone. On the other hand, Whitfield (1953) found in the unanaesthetized intact nervous system that rhythmic frequency variations in a stimulus would produce corresponding rhythmic activity at the cortex, suggesting a spatial frequency correlate.

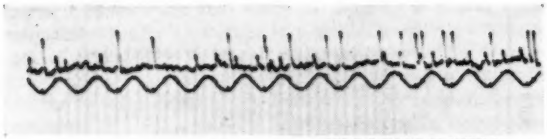
There is a good deal of evidence derived from experimental ablations to suggest that the auditory cortex is not essential for frequency discrimination, at least in quadrupeds, and Diamond & Neff (1953) in a preliminary report claim that the primary auditory area AI is not essential even for the recognition of tonal patterns in the cat.

4. Temporal Relations

Results derived from the responses of the auditory pathway to impulsive ("click") stimuli indicate that, as might be expected, a very large number of channels are excited by such a stimulus. The latency of the response increases from about 2 msec. at the cochlear nucleus to about 10 msec. at the cortex. Such clicks produce a discharge of several spikes with a mean interval of about 2 msec. or even less, compared with a mean interval of about 10 msec. or greater from fibres adapted to pure tones. The latency of the click response at the cortex increases as the stimulus intensity falls, varying between 6 msec. and 12 msec. (Davies, Erulkar & Rose, 1954); the number of spikes in the train also decreases with decreasing intensity. While some units respond with a latency within this range, a second group is found which has a very long latency of the order of 60–100 msec.

The higher mean discharge rates and great possibility of synchronism of pulses produced by impulsive stimuli are in

FIG. 3. RELATION OF NERVE IMPULSES IN A SINGLE UNIT IN THE TRAPEZOID BODY TO THE STIMULUS WAVEFORM



Hilali & Whitfield (1953) by permission of Journal of Physiology

Stimulus frequency: 100 cyc./sec.

A small fraction of the spike voltage has been superimposed on the stimulus record (lower trace) to show the relationship more clearly.

accordance with the known superiority of such stimuli over tones as a means of sound location, although it is surprising that temporal judgements are as good as they are, in view of the considerable variations in latency which are found. These variations can presumably partly be offset by the very large number of fibres activated, and hence the possibility of greater accuracy through the statistical comparison of the times of arrival over the whole array.

The first 10 msec. of the onset of a pure tone produces responses at all neural levels which are indistinguishable from those produced by clicks, so that the value of such onset tones in investigating frequency response would seem suspect on practical as well as on theoretical grounds. With pure tones there is a relation at low frequencies between the phase of the stimulating tone and the nerve impulse position. The " uncertainty of time of firing " of a nerve impulse is about 0.3 msec., so that the relation is completely lost for stimulus frequencies above 3,000 cyc./sec. at the second order neurone level, and is beginning to be inaccurate above 1,000 cyc./sec. At higher neural levels, owing to the interposition of further synapses and their variable delays, the maximum frequency is probably lower.

Owing to the non-regular nature of the neural discharge above the first-order level, and its comparatively low mean frequency, there will, in general, be less than one spike per cycle of the sound stimulus, although periodically two spikes may occur during one cycle; even so there is still a definite time relation maintained so that the spikes occur at corresponding points on the cycle (fig. 3). The electrophysiological findings are thus consistent with the known relation between phase and frequency in sound location.

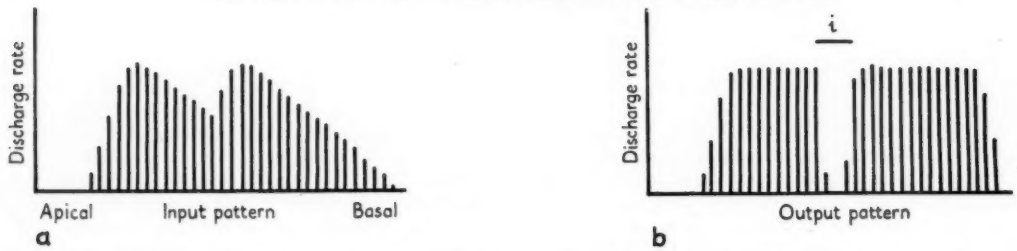
5. The Role of Inhibition

It was first demonstrated by Galambos (1944) that tonal stimulation could inhibit the discharge in a single unit, whether that discharge was " spontaneous " or produced by stimulation with a second tone. According to the experiments of Tasaki (1954), such inhibitory effects do not take place at the periphery, and the cochlear nucleus would appear to be the earliest site of such inhibition.

The role of inhibition at the cochlear nucleus in the auditory process has been discussed by Allanson & Whitfield (1956), who concluded that it was necessary to invoke such a process to account for the observed results, even when only single tones were considered. In many fibres of the efferent pathway " mutual inhibition " can be demonstrated (Whitfield, 1955). Either of two adjacent tones will produce a response in the unit, but when both are sounded together no response, or a reduced response, is obtained. The function of this inhibition would appear to be to maintain the separate identity of the blocks of fibres whose activity represents the two tones and to prevent their coalescence giving a resultant which would be interpreted as a single tone of intermediate frequency but greater loudness (fig. 4).

Erulkar (personal communication, 1955) has worked on the inhibition of the click response by tones, in single units at the inferior colliculus, and has found that there is, for each unit, a well-defined band of frequencies within which inhibition takes place, and whose width varies with intensity. The transition from absence of inhibition to complete inhibition takes place at each edge over a small percentage of the total band width and suggests the same distribution of activity as Hilali and Whitfield found at the trapezoid body.

FIG. 4. THE ROLE OF INHIBITION IN THE COCHLEAR NUCLEUS IN MAINTAINING THE IDENTITY OF TWO TONES SOUNDED SIMULTANEOUSLY



The method of representation is the same as that in fig. 2, and shows the patterns produced by simultaneous stimulation with two tones of frequencies equal to the characteristic frequencies of the two most highly excited fibres in fig. 4a. The " input " pattern represents the distribution of activity in the afferent pathway to the cochlear nucleus (auditory nerve), while the " output " pattern is the corresponding distribution in the efferent pathway from the nucleus (trapezoid body). Although the units in the region (i) are excited when either of the two tones is sounded alone, they are inhibited when both are sounded together. Were this not the case, the active arrays would coalesce to form a single block indistinguishable from that produced by a single, much louder, tone.

6. Summary

It has become evident that the nervous pathway plays a much more vital role in the hearing process than that of a mere transmitter of information. A considerable, though gradual, transformation of the spatio-temporal pattern of activity takes place as we ascend the system, so that frequency and intensity aspects of the stimulus are " concentrated " into particularly related fibre groups. Electrophysiological studies have allowed us to assess at various levels the parts played by neural interaction and by inhibition in this process, both for steady and for transient stimuli. Although the general pattern of activity is thus becoming clear, much still remains to be done, especially at the cortical level, where the behaviour and interrelationship of the several auditory areas which have been described in the literature remains obscure.

ACKNOWLEDGEMENT

I am grateful to the Editors of the *Journal of Physiology* for permission to reproduce fig. 3.

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COMPARATIVE PHYSIOLOGY OF THE OTOLITH ORGANS

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1 Morphology
2 The classical theory of otolith function
3 Oscillographic analysis of otolith function
  a The utriculus
  b The sacculus
  c The lagena
4 Conclusions
References

1. Morphology

The labyrinth of the vertebrates contains typically three otolith organs, situated in the three subdivisions of the vestibular cavity known as the utriculus, the sacculus, and the lagena. A sensory epithelium, the so-called macula, is covered by a membrane, which becomes associated in the majority of vertebrates with calcareous concretions (otoconia), or in the bony fishes with solid characteristically shaped otoliths. These calcareous formations serve to make the otolith membrane specifically heavier than the surrounding tissues and endolymphatic fluid. The otolith membrane so weighted is in close contact with the sensory hair processes of the underlying layer of secondary sensory cells, which, together with intercalated supporting cells, make up the epithelium of the macula. The strength and direction of the gravitational pull or of any other accelerating force is transmitted to the hair cells by commensurate deformations or dislocations of the otolith structures.

Phylogenetically, the otolith organs are by no means a vertebrate "invention". Their functional principle and the elements of their design are as old as the living cell. In plant cells and in the cell body of the Protozoa specifically heavier cell inclusions are very likely responsible for the elicitation of geotropic and geotactic responses. The statolith hypothesis of geotropism in plants postulates that starch grains may transmit the gravitational stimulus to the surrounding cytoplasm, and in the Protozoa the contents of food vacuoles are said to function in a similar way. Among the Metazoa, statocysts of various types are found in members of all the major phyla. Originally they were called otocysts, and an auditory function was attributed to them in view of their resemblance to the vertebrate otolith organs so closely associated with the organ of hearing. After the discovery of the equilibrium function of the vertebrate labyrinth, the term otocyst was generally abandoned in favour of the term statocyst, and with it the idea of an auditory function of all such organs.

2. The Classical Theory of Otolith Function

In the classical theory of otolith function an attempt was made to allot to each of the three otolith organs a particular orientation to which it was supposed to respond specifically by displacement of its otolith membranes. The parallel in spatial arrangement between the semicircular canals and the three maculae played an important but unfortunately misleading part in the analysis of labyrinth function. All this led finally to the elaboration of a theory of localization of static reflexes in mammals, propounded by Magnus and De Kleijn. The main gist of this was that the utricular otoliths subserved all bilaterally symmetrical labyrinthine reflexes, whereas the sacculi or at least parts thereof responded to bilaterally asymmetrical stimulation. All this was in fact later refuted and withdrawn by De Kleijn in the light of a whole series of experiments on a range of vertebrate animals including the mammals, from which it had become clear that the utriculus alone is potentially capable of controlling all static postural reflexes, and that the functional elimination of the sacculus has very little effect on equilibration. I have reviewed the relevant literature (Lowenstein, 1936, 1950). All this is by now ancient history, although the original theoretical speculations of Magnus and De Kleijn still enjoy a ghost-like existence in a number of otological textbooks.

3. Oscillographic Analysis of Otolith Function

Otolith organs are notoriously inaccessible, and their functional elimination without damage to other parts of the labyrinth is almost impossible in the majority of vertebrates, but especially in the mammals. It is therefore not surprising that no clear analysis of otolith function could be achieved until the development of the oscillographic technique made possible the recording of impulse responses from individual end-organs. The first recorded attempts with this technique were made by Ashcroft & Hallpike (1934) and by Ross (1936) in the frog. Ashcroft and Hallpike, recording action potential responses from the nerve from the saccule, found that rotating and tilting did not produce any excitation, and that the organ responded well to vibrations up to 512 cyc./sec. by firing in synchrony with the impulse frequency. Ross recorded the responses from both the anterior and the posterior main branch of the eighth nerve in the frog, and found that each contained fibres that respond to gravitational stimuli as well as fibres that respond to vibrational stimuli. The anterior branch innervates the utriculus and the sacculus apart from the horizontal and anterior vertical semicircular canals, the posterior branch, the lagena, the macula neglecta and the posterior vertical canal. Although Ross had no means of knowing the exact origin of the impulse discharges recorded from these complex branches, it is tempting to assume, in the light of later findings presently to be described (Ledoux, 1949; Lowenstein & Roberts, 1949, 1951), that the vibration responses in the anterior branch originated in the sacculus, those from the posterior branch originated in the papilla basilaris and macula neglecta, whereas the utriculus was responsible for the gravity responses in the anterior and the lagena for those in the posterior branch of the eighth nerve.

Absolute precision in the localization of the origin of labyrinthine responses was achieved when it was found possible in the isolated surviving labyrinth of the elasmobranch fish *Raja clavata* (thornback ray) to record activity from single functional units in the separate nerve branches innervating the various end-organs (Lowenstein & Sand, 1940; Lowenstein & Roberts, 1949, 1951). The elasmobranch labyrinth contains, apart from the semicircular canals, the usual three otolith organs, a well-developed utriculus, a large sacculus,

and a relatively small lagena, situated in a diverticulum of the sacculus. Each has an otolith composed of lime crystals deposited in a jelly-like matrix. The utricular macula is only partly covered by the otolith. A wing-like portion of it extending to the dorsal aspect of the recessus utriculi, and lacking any special covering structure, is known as the lacinia maculae utriculi. The saccular macula looks fairly uniform throughout and so does its otolith. The nerve supply of the macula, however, can be composed of derivatives of the anterior and posterior main branches of the eighth nerve, innervating the anterior and posterior portions of the macula respectively. In the mammals these two parts of the saccular macula are well distinguishable, as they are orientated in different spatial planes (de Burlet, 1929). No such morphological subdivision of the saccular macula is seen in the elasmobranch labyrinth, but, as will be shown, the anterior and posterior parts are functionally distinct. In the roof of the saccular cavity, near the insertion of the ductus canalis posterioris, lies the macula neglecta, a small patch of sensory epithelium without any covering structure.

a. The Utriculus

The electrophysiological analysis of the function of the various maculae (Lowenstein & Roberts, 1949, 1951) has yielded the following information. The main portion of the utricular macula responds to tilting from the normal position around all horizontal axes. It is therefore responsible for the control of reflexes both to fore and aft, and to lateral tilts, and all resultant combinations between them. There is also good reason to believe that it responds to linear accelerations forward and sideways, and in resultant directions. A single utriculus can register tilts and linear accelerations in opposite directions in a characteristic way. Take a single functional unit of the left utricular macula. In the normal spatial position of the skull this will be found to fire impulses at a moderate rate of between 10 and 20/sec. If this preparation is slowly tilted towards the left, the discharge frequency is found to decrease and to reach a minimum in or near the left-side-down position. A tilt to the right, however, leads to an increase in the discharge frequency with a maximum in the left-side-up position. In the upside-down position the discharge frequency resembles roughly that found when the head is upright (fig. 1). When the tilt is arrested in any given position in space, our end-organ will go on firing at a rate corresponding to the extent of the deviation from the normal. Although there is always a certain amount of adaptive change, the discharge frequency will remain characteristically different from that in the normal position. The behaviour of the sense organ just described is ideally suited to furnish positional information to the central nervous system. Not all receptor endings in the utricular macula, however, behave in this way. Others respond to any deviation from the normal with one and the same change in their activity irrespective of the direction of tilting. Following cessation of the tilting movement, the discharge activity of such organs speedily returns to the basic level. The information furnished by such receptor endings is therefore far from being concerned with posture. They are movement receptors delicately poised to signal the departure from certain positions as such. The term " out-of-position receptors " might be considered to describe their function most adequately.

When we consider that some of the positional receptors have their maximum discharge frequency either in the side-up and nose-up, others in the side-up and nose-down position, and also that there are transitional types between the positional and the out-of-position receptors; and finally when we keep in mind that there may be a considerable number of receptors which keep on firing at a steady rate of impulse discharge which is practically unaffected by positional changes, it becomes clear that the utricular macula is far from being a uniform structure. A variety of differently responding receptor endings may be assumed to contribute to a point-by-point report on the mechanical relationship between macula and otolith during a displacement or deformation of the latter by positional changes of the head. Other receptor endings may furnish a background of unchanging impulse flow which very likely is the origin of the continued tonus production by the macula which has been observed to go on even after removal of the otolith.

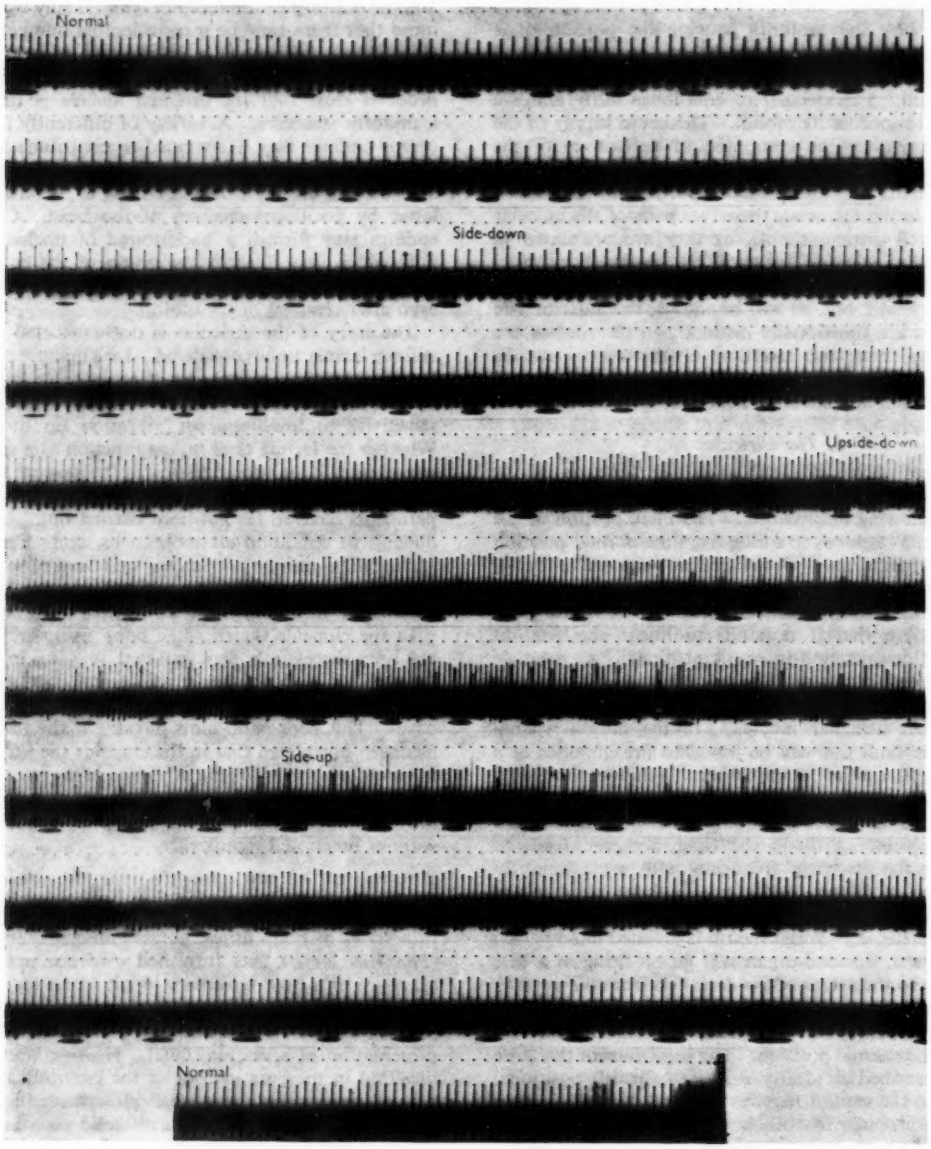
The story of the utriculus is not exhausted yet. There is still the lacinia to be considered. Lacking any covering structure, it is entirely composed of receptor endings unaffected by positional changes. However, it responds with startling sensitivity to low-frequency vibration up to 120 cyc./sec. Whether the lacinia does in fact function as a sound receptor has to remain an open question in the absence of a thorough analysis of sound perception in elasmobranchs. Such experiments have so far not been carried out. It is extremely difficult to condition elasmobranchs, and—like many other marine fish—they do not recover well from surgical operations such as would have to be carried out for the functional elimination of the labyrinth as a whole or in part.

In the clupeids (herring-like bony fish), sound perception has been successfully demonstrated, and it is extremely likely that the air bladder is involved in sound conduction to the labyrinth with which it is connected by strands of fibrous tissue. This connexion aims directly at the utriculus, and it has been postulated that in the clupeids the utriculus may be the auditory organ. There is no sign in the ray of any association of the utriculus with possible pathways of sound, and it may well be that the vibration sensitivity of the lacinia is here without functional significance.

b. The Sacculus

The work of von Frisch and his school on sound perception in a series of bony fishes, notable among them the minnow (*Phoxinus laevis*), has furnished evidence not only for an existence of a high sensitivity to sound up to 7,000 cyc./sec., but also for pitch discrimination up to a limit of 800 cycles as acute as a minor third absolute, and less than a semitone, on presentation of a warble signal. Hearing was shown to be localized in the pars inferior of the labyrinth which consists here of the sacculus and lagena well separated from the rest of the labyrinth and equipped with solid otoliths of elaborate shape. Especially the saccular otolith, the sagitta, has striking wing-like extensions and lies delicately suspended over the saccular macula. The air bladder is connected with the posterior part of the sacculus by means of a chain of three Weberian ossicles which may well serve as sound conductors linking the air bladder, acting as a resonator, with the sound receptors in the saccular macula. There is experimental evidence for the impairment of auditory acuity after elimination of these ossicles. Whether the lagena, too, is concerned here with sound perception is not certain, but it was shown that the whole pars inferior cannot, after the isolated bilateral removal of the pars superior (utriculus and semicircular

FIG. 1. CONTINUOUS RECORDIOF A TWO-UNIT RESPONSE FROM THE UTRICULUS MACULA OF *RAJA CLAVATA* TO A FULL-CIRCLE LATERAL TILT



Lowenstein & Roberts (1949) by permission of Journal of Physiology

Time marker at top of record: 24/sec.
Rotation signal at bottom: one gap every 3°
Speed of rotation: 10°/sec. approximately

canals), elicit a single gravity response of fins or eyes, whereas its isolated bilateral elimination does not in the least interfere with equilibration (von Frisch & Stetter, 1932; Lowenstein, 1932). It is clear, therefore, that the sacculus at least must be considered to have a purely auditory function in this group of fishes.

In the elasmobranch the situation is complex. Nerve fibres from about the anterior two-thirds of the saccular macula yield pronounced low threshold responses to vibration up to 120 cyc./sec., with clear synchronization between signal and impulse frequency (Lowenstein & Roberts, 1951) (fig. 2). Positional changes are without effect on these end-organs.

FIG. 2. RECORDS OF VIBRATIONAL RESPONSES FROM THE SACCULUS OF *RAJA CLAVATA*, SHOWING THE CONSTANT PHASE RELATIONSHIP BETWEEN MICROPHONIC WAVES AND SUMMATED SPIKE POTENTIALS

Lowenstein & Roberts (1951) by permission of Journal of Physiology

Record 1: stimulus frequency, 110 cyc./sec.
Record 2: stimulus frequency, 100 cyc./sec.
In both records a number of sweeps are superimposed.

Similarly, the macula neglecta responds to the same range (if anything still more sensitively than the sacculus). Here, too, the absence of good behaviouristic confirmation makes it difficult to be certain whether these end-organs in fact serve for hearing. However, an interesting piece of morphological evidence makes this much more likely than in the case of the lacinia. There appears, in fact, to be provision for sound conduction towards the sacculus. Above it, in the roof of the skull, is found a membrane-covered perforation known as the fenestra of Scarpa. It is quite possible that this structure, in conjunction with the adjoining ductus endolymphaticus, represents a sound-conducting mechanism. Sacculus and macula neglecta could therefore be considered so-called perilymphatic sense endings in the sense of de Burlet (1935), who maintained that potentially all labyrinthine end-organs might be considered capable of vibration reception, and that whether they do in fact have an auditory function depends entirely on the degree and nature of exposure to impingent sound. Conversely, the absence of vibration sensitivity in other end-organs may well be due to their effective insulation against sound. This argument may bring to mind the fact that even semicircular canals can become sensitive to sound when they are artificially exposed to it, as is the case, for instance, after a fenestration operation. This does not, however, mean that sound perception belongs to the natural range of functions of the semicircular canals.

The posterior third of the saccular macula yields positional responses of exactly the same type and functional range as the utriculus. It is strange to find that the same macula behaves so differently in two of its parts which are not morphologically distinguishable in any way. The posterior part may be lodged more firmly in the cartilaginous otic capsule, being thus somewhat shielded from vibrations, whereas the portion of the otolith situated in the anterior end is free to vibrate relative to the surrounding tissues. However, the otolith mass is continuous without any obvious subdivision or change in density or texture.

c. The Lagena

No responses to vibration are obtained from the lagena immediately adjoining the positional part of the sacculus. Its responses are also positional, but differ essentially from those of the utriculus and sacculus. The responses show greater uniformity throughout the macula, and as a rule the receptor units have a sharp maximum of impulse frequency in or near the normal spatial position of the skull. This feature is so striking that it appears as if the chief function of the lagena is to mark the normal position, and signal any deviations from it by a sharp fall in activity, and the return to it from any direction with an equally steep rise. It was therefore proposed to interpret its function as that of an " into-level " receptor. The lagena receptors respond to both lateral and fore-and-aft tilts, and one and the same end-organ can be found to be equally affected by both. These functional properties of the elasmobranch lagena tally well with certain observations in the Amphibia. McNaughton & McNally (1946) reported that they succeeded in the isolated elimination by nerve cutting of the lagena in the frog. The unilateral operation resulted in a slight upward tilt of the head on the ipsilateral side, and the effect of the bilateral operation was a slight unsteadiness of the head on cessation of movement. On the other hand, the possession of two intact lagenae, in the absence of all other labyrinthine end-organs, left the frog with a righting capacity distinctly superior to that of a totally labyrinthectomized animal. From this McNaughton and McNally concluded that the lagena has an equilibrium function and that it may be considered to antagonize the activity of the utriculus. This fits well with a description of this organ as an into-level receptor, and with the electrophysiological findings in the ray. Similarly, it had been reported that the removal of the pars inferior of the labyrinth in birds (this contains here the sacculus, the cochlea, and the lagena) slightly affects certain eye reflexes (Benjamins & Huizinga, 1927, 1928). The reflexes in question are the rotary eye deviations occurring in fore-and-aft tilts.

Almost insuperable difficulties stand in the way of a separate elimination of utriculus or sacculus in the mammals. One successful attempt to eliminate the sacculus surgically by interruption of its nerve supply has been reported by Versteegh (1927). He observed that in rabbits this operation leaves the equilibrium reflexes intact; however, he, like Benjamins and Huizinga, reports a slight impairment of the rotary eye reflexes on fore-and-aft tilting. The possibility of slight damage to the pars superior was admitted by Versteegh as a possible reason for this reflex deficiency. The chief point, however, is that it is very unlikely that the mammalian sacculus has a significant share in the postural control. Whether this means that it has an auditory function accessory to that of the cochlea is unfortunately an open question. It is interesting in this connexion to consider what this function might be. We know from the experiments in fishes that an otolith organ can signal not only the presence or absence of sound, but can furnish information concerning its intensity and frequency. Frequencies are signalled by synchronization of the impulse with the signal frequency, and this appears to occur up to an upper limit of roughly 800 cyc./sec. in fishes (with action potential responses in the elasmobranchs, up to 120 cyc./sec. only, and saccular microphonics up to 780 cyc./sec.). It is very likely that this low cut-off is largely due here to the unnatural mode of stimulus transmission in the isolated preparation of the labyrinth, and not much physiological significance should be attached to it.

In the mammal, signal-synchronized impulse discharges have been observed in the eighth nerve up to 4,000 cyc./sec. (Galambos & Davies, 1943, 1944). It is not unlikely that the saccular macula may be responsible at least in part for this

Continued at foot of page 114

PERIPHERAL MECHANISMS OF EQUILIBRIUM

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1 The labyrinth as an organ of equilibrium
2 The mode of function of labyrinthine sense endings
3 The semicircular canals
4 Mechanical aspects of canal function
5 Semicircular canals and otolith organs
References

The resting posture of a vertebrate is characteristic for the species and is maintained by general tonus distribution to the anti-gravity muscles, and by specific righting and other compensatory reflexes in response to passive dislocation and active movement.

1. The Labyrinth as an Organ of Equilibrium

The labyrinth is not by any means the only organ concerned with the control of equilibrium. Tactile receptors in the integument and deep pressure receptors, the mechano-receptors in muscles, tendons and joints (proprioceptors in the narrow sense of the word), and finally, of primary importance, the eye, are all contributing to it.

The contribution of the labyrinth to the peripheral control of equilibrium is probably most important in free swimming and flying vertebrates, i.e. in the fishes and the birds. In the squatting amphibia and reptiles it may be subordinate, whereas in the long-limbed quadrupeds and bipeds the extent of its contribution depends very largely on the mode of life of the organism, on the degree of visual orientation, on the type of locomotion, and so on. In man bilateral lack of labyrinthine function can usually be perfectly compensated for, except under the conditions outside the range of normal human activities such as diving and swimming under water. Labyrinthine equilibrium rests on a dual sensory mechanism: the perception of linear acceleration and movement at constant speed, on the one hand, and the perception of angular acceleration on the other. Both functions are based primarily on the deformation of the hair processes of secondary sensory cells situated in the sensory epithelia of otolith maculae and of the cristae of the semicircular canal ampullae respectively. Certain aspects of the principles involved in the mode of functioning of the otolith organs are dealt with in the article on their comparative physiology in this number of the Bulletin.[1] It will be necessary, however, in the present context to consider some functional problems which are common to the hair-cell receptors in all types of labyrinthine end-organs.

2. The Mode of Function of Labyrinthine Sense Endings

One of the facts that have emerged from the electrophysiological analysis of the equilibrium function of labyrinthine receptors, chiefly in the elasmobranch fish *Raja clavata* (Lowenstein & Sand, 1940b; Lowenstein & Roberts, 1949), is the existence of what has been described as a spontaneous discharge activity. A considerable proportion of the sensory cells in the cristae and maculae are constantly active. Their impulse discharge is extremely regular, with deviations from the average frequency as small as 4% (Groen, Lowenstein & Vendrik, 1952), and in isolated preparations end-organs can be observed to fire for hours at an unchanging frequency. Naturally such a discharge activity emanating from a labyrinth at rest in its normal spatial position appears to be spontaneous. It has been so described. The term spontaneous does, however, invite semantic scrutiny, and there emerge different degrees of validity of the term, when the actual

[1] See Lowenstein, p. 110 of this number of the Bulletin.—ED.

COMPARATIVE PHYSIOLOGY OF THE OTOLITH ORGANS

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signal-synchronized transmission of sound stimuli. The evolution of the cochlea may thus be understood as a means of extending pitch discrimination beyond the limits set to signal-synchronized transmission by the refractoriness of the nerve fibre.

4. Conclusions

Otolith organs have thus been shown to have potentially a dual function. They can serve as pure gravity receptors, with an associated function as receptors for linear movement and centrifugal force, or as receptors for vibrations, or both—as in the case of the elasmobranch sacculus. In fact the otolith organ is fundamentally designed as a receptor for linear accelerations of all kinds. Whether a given otolith organ is used by the animal for one or other purpose depends entirely on its manner of association with auxiliary structures such as sound conductors, acoustic windows; or on the type of suspension or lodgement within the otic capsule. The elasmobranch labyrinth is sufficiently generalized to give evidence of all these potentialities side by side.

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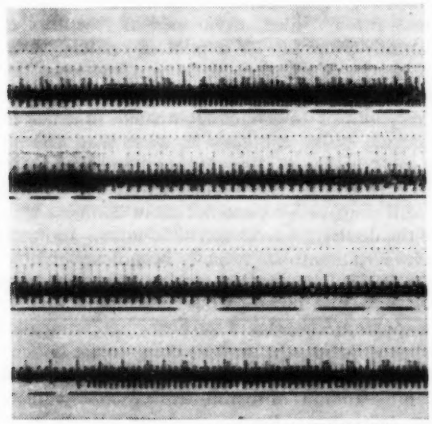
physiological conditions are taken into account. In otolith-bearing maculae an unstimulated state may hardly ever exist unless in a spatial position where the gravitational pull on the otolith fails to deform any of the hair processes. Judging from the presence of continuous impulse activity in the sense endings of the lacinia of the utriculus in the elasmobranch fish *Raja clavata* (Lowenstein & Roberts, 1951), it may be safe to assume that, as this part of the macula does not carry an otolith, macula cells in general can fire impulses in the absence of gravitational stimulation. A similar situation exists in the case of the sensory cells of the cristae of the semicircular canals. There the hair cells are stimulated by inertia movements of the gelatinous cupula in which their hair processes are embedded. The cupula deformations take place only during angular accelerations or decelerations of the labyrinth. Yet at rest many of the cells discharge continuously at a moderate rate up to 20 impulses/sec. Whatever the cause of this " basic " activity, it is so widespread among the sensory cells of the labyrinthine end-organs that there is every reason to assume that it is an essential part of the functional behaviour of these receptors (Lowenstein & Sand, 1940a, 1940b; Ledoux, 1949; Lowenstein & Roberts, 1949, 1951).

3. The Semicircular Canals

A receptor ending in the horizontal semicircular canal of the ray, firing basically at 20 impulses/sec. when the labyrinth is at rest, will increase its discharge rate on ipsilateral, and decrease it ultimately to zero on contralateral, acceleration. These two directions of acceleration bring about ampullopetal and ampullofugal endolymph movement respectively. Sudden arrest of rotary movement results in an increased impulse discharge after contralateral, and a reduction in discharge rate after ipsilateral, acceleration (fig. 1). In the vertical canals, ampullofugal endolymph movement is stimulatory, and ampullopetal movement inhibitory. This is in absolute agreement with the picture of reflex responses demonstrated by Ewald in his classical experiments with direct stimulation of the semicircular canals by means of a pneumatic stimulator. Apart from the type of end-organ just described, others are found in the canal cristae. Some are silent at rest and discharge during ipsilateral and after contralateral acceleration only, with correspondingly opposite after-effects. They are therefore truly uni-directional as opposed to the bi-directional majority. Yet other end-organs are found active at rest at frequencies above the usual " spontaneous " level. They are hardly influenced by rotations at all. Now it is interesting that these are not absolutely discrete types of fundamentally different receptors. All transitional stages can be found, among which the bilateral receptors take up a central position in so far as many of them can be found to be equally sensitive to ipsilateral and contralateral rotation.

A unified functional picture emerges when one plots the stimulus response relationships in the various receptors. This yields a curve (fig. 2) which resembles the so-called characteristic curve describing the behaviour of an electronic valve (fig. 3), stimulus intensity and resulting action potential frequency corresponding with signal voltage at the grid (fig. 3, V_G) and anode current (fig. 3, I_A) respectively. The resting discharge can be likened in this case to the anode current determined by a certain grid bias voltage. The curve in fig. 2 was plotted from values obtained in an analysis of the action potential responses from a three-unit preparation in the

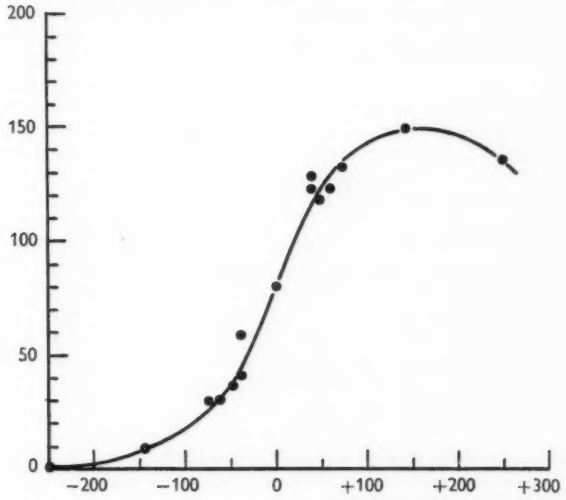
FIG. 1. OSCILLOGRAPHIC RECORDS FROM THE NERVE BRANCH OF THE LEFT HORIZONTAL AMPULLA OF THE RAY, SHOWING THE RESPONSE OF A SINGLE UNIT TO ANGULAR ACCELERATION TO THE LEFT (A) AND TO THE RIGHT (B)



Lowenstein & Sand (1940b) by permission of the Royal Society

The lower strip of each pair is the continuation of the upper.
Top record shows time in 0.05-sec. intervals.
Single line at bottom records accelerations. Successive gaps in the line mark the revolution of the turn-table through 12°.

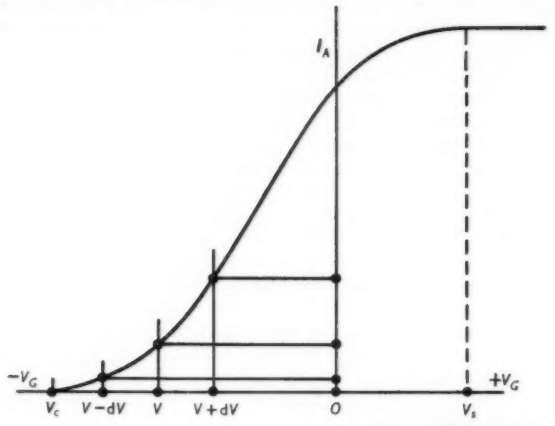
FIG. 2. CHARACTERISTIC CURVE OF ACTION POTENTIAL FREQUENCY PLOTTED AGAINST IMPULSE STIMULI, USING A THREE-UNIT PREPARATION OF THE HORIZONTAL SEMICIRCULAR CANAL OF THE RAY



Groen, Lowenstein & Vendrik (1952) by permission of Journal of Physiology

Abscissae: stimulus strength (rotational velocity (deg./sec.) at "stop")
Ordinates: response frequency (action potentials/sec.)
(The mechanical impulses are produced by sudden stopping of steady speed rotations.)

FIG. 3. CHARACTERISTIC CURVE OF A TRIODE VALVE



Groen, Lowenstein & Vendrik (1952 by permission of Journal of Physiology

Abscissae: grid voltage (V_G)
Ordinates: anode current (I_A)

V: grid bias setting on non-linear part of characteristic. Note unequal effects on anode current of equal increase and decrease in grid voltage ($+ dV$; $- dV$).

V_c and V_s: opposite extremes of grid bias

horizontal semicircular canal of the ray, using accelerations covering a wide range of intensities (here expressed as mechanical impulse in degrees per second). It can be seen that the end-organs' response is linear over a considerable range of stimulus intensities.

In comparing the various types of ending described above with the behaviour of this preparation, one is struck by the possibility that their behaviour may be understood by making the assumption that, in the resting stage, they occupy different positions on such a characteristic curve. This might be caused by a shift in the conditions in that part of the receptor responsible for the translation of the mechanical stimulus into the impulse discharge, viz., in the membrane of the dendritic fibres surrounding the sensory cell. It may be assumed that, in conformity with the electronic valve model, a biasing condition may shift the working point of a certain cell up and down the characteristic curve. A symmetrical bi-directional receptor would lie in the middle of the straight part of this curve, and equally strong but oppositely directed rotary stimuli would evoke an equal increase and decrease of discharge rates from the basic level. The receptor biased to have its working point near the bottom of the curve (fig. 3, V_c) would be silent at rest, and would show a response to rotation in one direction only, and also would have a relatively high threshold. At the top of the curve (fig. 3, V_s) would be found those receptors which fire at a high rate and do not respond at all to a considerable range of stimulus intensities.

In an investigation into the effects of galvanic polarization on the impulse discharge from labyrinthine sense endings (Lowenstein, 1955), it could in fact be shown that, by application of d.c. currents made to flow through the sensory nerve towards the sense endings and from the sense endings through the sensory nerve towards the central nervous system, their discharge activity could be quantitatively "biased" in the manner suggested. The effects of the polarizing potential and the responses to rotation could be made to sum arithmetically with one another, and by a shift of its working point a symmetrically responding organ could be converted into an asymmetrical one, and vice versa.

So far as the sum total of different signals from a whole crista is concerned, the composite picture may be somewhat like this. A considerable amount of afferent discharge is pumped into the vestibular centre by the "spontaneously" firing units. The majority of these are delicately poised to react to the slightest cupula movement by a change in the rate of an already existing discharge activity. Peripherally, therefore, a threshold hardly exists at all, and it can be assumed that, whatever the threshold for reflex responses to rotation may be, this is predominantly of central nervous origin. At rest the anti-gravity muscles and other effector muscles involved in vestibular reflexes, notably the eye muscles, are under constant tonic influence from such canal receptors. This was confirmed in elimination experiments on the pike (Lowenstein, 1937), where it was possible by operative interruption of the nerve supply to one of the horizontal canals of a blinded fish to create a lasting asymmetry in the tonus of the horizontal eye muscles (rectus internus and externus). The resulting eye deviation towards the side operated on diminished in time by central nervous compensation, but never disappeared completely. The semicircular canals are thus an important source of vestibular tonus. This was first postulated by Ewald. Later, however, under the impact of the teaching of the Magnus school, it became customary to ascribe this role almost exclusively to the otolith organs.

The bi-directional symmetrical receptors then initiate the vestibular responses to rotation by bringing about smooth adjustments in the tonus of the effector muscles concerned (compensatory movements of eyes and limbs). In the case of the eyes, compensatory deviation is followed by the oppositely directed nystagmic jerk. This is of central nervous origin, and is probably triggered off by maximal excitation of the eye-muscle motor centres. The responses to rotation are graded and may be assumed to depend on the total change in discharge frequency in the canal receptors and also on the total number of receptors affected by rotation. This is where the "spontaneously" silent asymmetrical receptors come into the picture. They furnish a pool of relatively less sensitive sensory units which are gradually recruited when the stimulus intensity rises. Direct evidence for this recruitment is obtained in preparations of the semicircular canals in which the recording electrode makes contact with more than one nerve fibre. Under such conditions it can be seen that, with an increase of the stimulus, in this case angular acceleration, the originally single unit preparation becomes more complex by the intrusion of the discharges from recruited units. Indirect evidence for such recruitment has recently been described by Hallpike & Hood (1953), who carried out turn-table tests with human subjects during an investigation into fatigue and adaptation of the cupular mechanism of the horizontal semicircular canal. In this investigation the authors come to the conclusion that the observed adaptation phenomena strongly point to a considerable amount of recruitment of sensory units at higher stimulus intensities. Recruitment can also be well demonstrated in preparations of the elasmobranch labyrinth subjected to d.c. polarization. Apart from raising the basic discharge rate in bi-directional endings, an increase in the strength of ascending polarizing current (flowing through the nerve towards the sense ending) brings in, one after the

other, previously silent units of higher threshold. These units often adapt themselves rather rapidly, and fire over a limited range of stimulus intensities only.

When Ewald first sketched out a picture of the mode of function of the semicircular canals he postulated bi-directional reactivity, but pointed out that in so far as the horizontal canal is concerned, ampullopetal (i.e. excitatory) endolymph movement produced stronger responses than the ampullofugal or inhibitory endolymph movement. The reverse was found to be true in the case of the vertical canals. Ewald's postulate of the preponderance of the stimulatory effect over the inhibitory one is in line with the results of the oscillographic analysis, and it appears that the fact of recruitment of additional receptors on ipsilateral rotation, and the absence of a similar possibility on contralateral acceleration in the horizontal canal, would furnish a basis for such a preponderance even if the majority of bi-directional receptors were symmetrical in their responses to rotation in opposite directions.

Hallpike & Hood (1953), impressed by the absence of such preponderance in caloric tests with warm and cold water, and by certain aspects of sensory adaptation to constant acceleration, formulated an hypothesis according to which the resting discharge originates in special endings situated at the base of the cupula and supplied by thin nerve fibres. Two distinct types of large hair cells innervated by so-called colossal fibres, and situated on either side of the crista ridge, are held to be " spontaneously " silent and to come into play, the one on ampullopetal, the other on ampullofugal endolymph displacement. Synaptic contact is assumed to be established between these two types of elements, either in the crista itself or in a vestibular ganglion, and the large hair cells are said to be responsible for the acceleration or inhibition of the rate of impulse discharge from the spontaneously active end-organs. In support of this rather complicated functional picture, Hallpike and Hood refer to experiments by Gernandt (1949). This author found that, in experiments on the cat, the majority (over 80%) of single-unit responses recorded from second-order neurones in the vestibular nerve are of the spontaneously active and bi-directional type described by Lowenstein & Sand (1940b) (type I). However, two other spontaneously active functional types were encountered. One showed an excitatory, the other an inhibitory response to acceleration in both directions (types II and III).

I have never come across such units in the elasmobranch labyrinth except in over-age preparations in which the cupula in the isolated labyrinth had become flaccid, or in preparations in which directiveness had disappeared after loss of endolymph following upon the opening into the vestibular cavity by canal sectioning or by accidental damage. It must, however, be borne in mind that Gernandt's records were obtained from second-order neurones, and his findings would be compatible with the assumption that these neurones may have been in synaptic connexion with pathways derived from the labyrinth opposite. That the mammalian labyrinth itself should be so fundamentally different in its mode of function from the labyrinth of the lower vertebrates appears to be very unlikely.

4. Mechanical Aspects of Canal Function

Let us now consider some of the mechanical aspects of the function of the semicircular canals. For a long time the existence of the cupula was a matter of doubt. It was said that it was a fixation artifact, and the sensory hairs were considered to end freely in the endolymph. This question was settled by Steinhausen's discovery of a means of observing the living cupula and its movements in the ampulla in response to natural rotatory stimulation (Steinhausen, 1931). Up till then, it was believed that endolymph flow could be of only very short duration, however strong the stimulus, and a maximal period of endolymph movement of about 0.5 seconds was calculated on the basis of the dimensions of the human labyrinth. The prolonged after-effects, such as post-rotatory nystagmus and vertigo lasting for more than 30 seconds, were held to be entirely of central nervous origin. Steinhausen and later Dohlman (1935), however, showed that the cupula behaved like an over-critically damped pendulum. The cupula fills the ampullary space reaching up to the ampullary wall opposite the crista, and during deflection moves in it with a close fit after the fashion of a revolving door. It also became clear that the cupula is an elastic structure and endowed with a restoring force which would make it return on its own from passive deflections. The time taken for this return depends on a number of structural parameters, and of course on the extent of the initial deviation. It is of an order of 20–30 seconds in the pike. This fits in well in man with the time-course of post-rotatory after-effects, such as post-rotatory nystagmus and false rotatory sensations during angular movement at constant speed. It also agrees with the time-course of the post-rotatory inhibition of the impulse discharge after ipsilateral, and post-rotatory rebound after contralateral, acceleration. It has become clear that it is unnecessary to invoke central nervous mechanisms for the interpretation of the reflex responses. The mechanical properties of the cupula pendulum can account for it all.

The picture thus appears to be this: during acceleration from rest, the cupula is deflected and the associated increase in discharge rate of the receptor cells at any given moment is proportional to the rotational speed at that moment. Any change in speed or acceleration will produce a change in the discharge activity. Constant speed rotation does not affect the cupula.

As pointed out above, the magnitude of deflection at any moment during the process of acceleration is a function of the angular velocity at that moment. Subjectively we experience sensations of change of velocity only. Strictly constant speed rotation on a perfectly smooth turn-table does not evoke any sensation. After the initial acceleration from rest has brought about a deflection of the cupula associated with the sensation of accelerated rotary motion, the continuation of the turn-table rotation at constant speed is accompanied by a gradual return of the cupula to its resting position. The sensation evoked by this cupula movement is one of being rotated at a gradually decreasing speed, and the sensation will last until the cupula is at rest. Nothing further will be perceived if the turn-table goes on to be rotated at constant speed. If, however, the turn-table is now stopped relatively suddenly, the cupula will be deflected in the opposite direction and this will evoke a corresponding subjective sensation of a virtual movement in the direction opposite to that of the preceding turn-table rotation. This sensation will be proportional to the magnitude of deceleration and will be followed by a decay of subjective movement lasting for the time taken for the cupula to return to its resting position once again. The post-rotatory eye nystagmus is a well-known phenomenon accompanying these subjective sensations.

van Egmond, Groen & Jongkees (1949) demonstrated that normally the semicircular canal acts as an impulsometer, comparable in principle to a ballistic galvanometer. Normal active movements are reflexly controlled in such a way that after-sensations are prevented. Excitation of the canal gives rise to a very active subjective estimate of the angle of turning, provided that (in man) the movement does not last longer than 3 seconds. Groen, Lowenstein & Vendrik (1952) were able to subject the impulse activity of the semicircular canal endings in the elasmobranch labyrinth to a mechanical analysis, and elaborated a differential equation describing the behaviour of the cupula-endolymph system. Their results are in very close agreement with the theoretical deductions from tests on human subjects.

5. Semicircular Canals and Otolith Organs

As a first approximation it is correct to say that the mode of functional collaboration between semicircular canals and otolith organs rests on the fact that the semicircular canals react only to angular accelerations, whereas the otolith organs are sensitive to angular accelerations, to rotation with constant speed, and to linear acceleration. It has been usual, in the past, to describe the semicircular canals as organs of the dynamic sense, and the otoliths as static sense organs. The way in which the utriculus responds to constant-speed rotation (tilting) is shown in fig. 1 of the article on the comparative physiology of otolith organs in this number of the Bulletin.[a] Constant-speed rotary movements are, however, of rare occurrence in the normal behaviour of any vertebrate, and it may therefore be said that the control of most angular movements is the domain of the semicircular canals. It is different so far as linear movement is concerned. Despite a number of claims to the contrary, there is no reason to assume that semicircular canals respond to linear accelerations of the order of magnitude normally encountered by the organism. Centrifugal force is a linear acceleration which may be of biological importance in bird flight. Reflex responses to centrifugal force are of a static nature, summing vectorially with gravity responses. Like the latter they are controlled by the otolith organ.

Phylogenetically the collaboration between semicircular canals and otolith organs goes back to the cyclostomes where, in *Myxine*, we find two vertical ampullae sharing a single canal and a single undivided otolith bearing macula. The collaboration between the easily motile cupula organ and the more inert otolith thus appears to be fundamental, although potentially the otolith organ could deal with all dynamic and static aspects of equilibrium.

[a] See Lowenstein, p. 110 of this number of the Bulletin.—Ed.

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LOUDNESS RECRUITMENT

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1 Loudness recruitment: the phenomenon
2 The anatomical basis of loudness recruitment
3 Loudness recruitment and its relationship to adaptation
4 Loudness recruitment and speech intelligibility
5 Technique of loudness recruitment tests with especial reference to the measurement of the intensity difference limen and pure tone audiometry
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The loudness recruitment phenomenon was first described by Fowler (1936). Its occurrence in subjects suffering from certain varieties of nerve deafness has since been abundantly confirmed (Steinberg & Gardner, 1937; Huizing, 1942; de Bruine-Altes, 1946). Such subjects, although unable to understand speech at ordinary levels of intensity, complain of excessive loudness if these levels are exceeded. The investigation of loudness recruitment is best undertaken in cases of monaural deafness by means of the binaural balancing technique first described by Fowler. A brief description of the test follows.

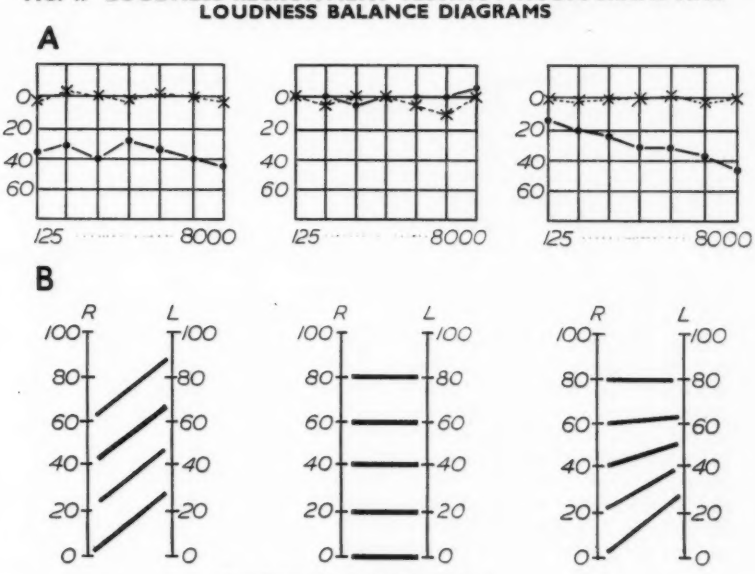
1. Loudness Recruitment: the Phenomenon

The subject wears a pair of telephone receivers each supplied from a separate pure tone audiometer, or preferably from a single audiometer with arrangements for independent adjustment of the intensities. The frequency of the sound stimulus is the same in each receiver, and the tester switches it alternately to left and right.

Two series of intensity levels are ascertained and marked upon the ladder diagrams, one for the right ear and one for the left, each intensity for the right ear being connected with the level for the left ear found to give a sensation of equal loudness. Figures shown in fig. 1 represent stimulus intensities in decibels above the normal threshold. In the centre are shown the results obtained in a normal subject, with the rungs of the ladder lying horizontally from bottom to top. On the left and right are shown the results obtained in two typical cases of unilateral deafness. On the left the deafness is of the conductive type due to middle ear disease; on the right, of the nerve type due to Ménière's disease of the labyrinth. In each case the test frequency selected is 1,000 cyc./sec., at which point the audiogram shows a threshold shift for the affected ear of 30 db. The result obtained in the case of conductive deafness indicates that the loss of sensitivity of the affected ear, 30 db. at threshold, remains constant at 30 db. throughout the entire intensity range. A very different result is obtained in the case of nerve deafness. Whereas the balancing points at threshold show the same displacement, 30 db., for the deaf ear, this becomes progressively less with increasing intensity until, at 80 db., equal intensities at the two ears evoke equal loudness responses. In other words the deafness of the affected ear present at threshold disappears at higher intensities, and this in its simplest terms constitutes the phenomenon of loudness recruitment.

A more conventional graphic representation of these results was used by Fowler, and is given in fig. 2. Sound intensities above the normal threshold are plotted on the vertical axis for the normal ear and on the horizontal axis for the deaf ear. Equal loudness levels for the two ears are plotted on this chart as a series of points. The line AB represents the result in a normal subject; CD that obtained in a case of conductive deafness. The deafness remains constant at higher intensities, and CD is thus parallel to AB. The curve EF is obtained in a case of nerve deafness with loudness recruitment. The

FIG. 1. LOUDNESS RECRUITMENT TESTING: AUDIOGRAMS AND LOUDNESS BALANCE DIAGRAMS



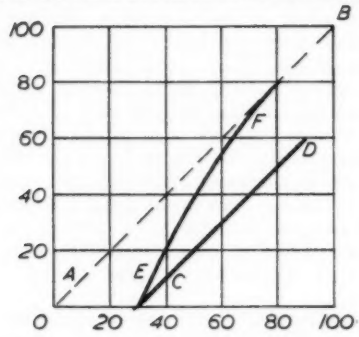
Test frequency: 1,000 cyc./sec.

Centre: results in normal subject
Left-hand: subject with conductive deafness of left ear
Right-hand: subject with nerve deafness of left ear

A. Audiograms:
Abscissae: frequency (cyc./sec.) (logarithmic scale)
Ordinates: hearing loss (db.)
●———● left ear (*L*)
x- - - - - - - - x right ear (*R*)

B. Loudness balance diagrams (stimulus intensities in db. above the normal threshold)

FIG. 2. LOUDNESS BALANCE TESTS : RESULTS IN NORMAL SUBJECT AND IN SUBJECTS WITH UNILATERAL CONDUCTIVE AND NERVE DEAFNESS



Abscissae: sound intensities for deaf ear (db.)
Ordinates: sound intensities for normal ear (db.)

AB: normal subject
CD: subject with conductive deafness
EF: subject with nerve deafness

deafness is progressively eliminated at higher intensities, and the line EF thus approaches and finally coincides with the line AB.

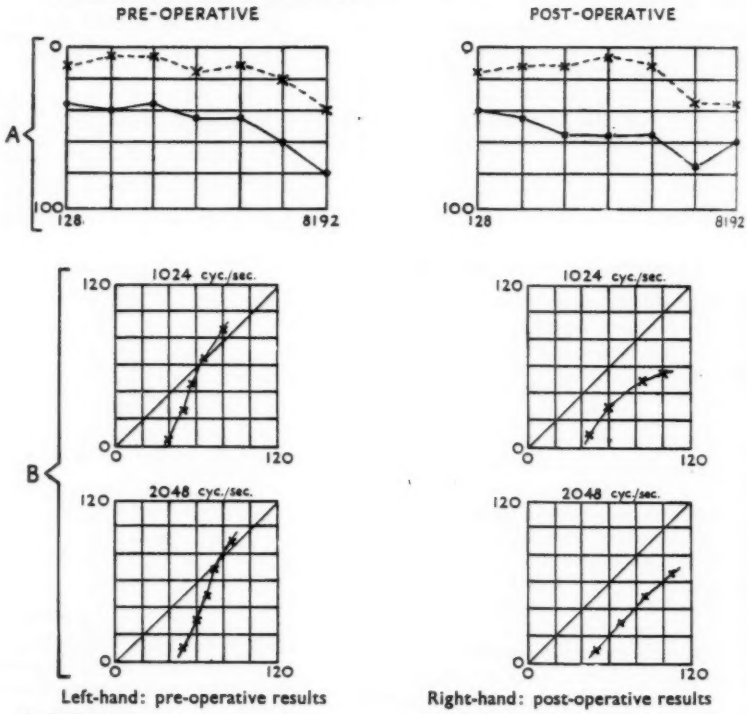
2. The Anatomical Basis of Loudness Recruitment

Loudness recruitment was first believed by Fowler and others to be characteristic of a wide variety of disorders of the cochlear neural apparatus including the cochlear nerve and its central connexions. The later work of Dix, Hallpike & Hood (1948) has, however, made it clear that the phenomenon is characteristically associated with pathological processes involving the hair cells of Corti's organ.

This conclusion was based upon comparative clinico-pathological studies of two well-known disorders of the eighth nerve system: firstly, Ménière's disease, in which the pathological process is known to affect the sensory cells of Corti's organ; secondly, degeneration of the cochlear nerve fibres due to tumours of—or affecting—the eighth nerve. Loudness balance tests were carried out by means of Fowler's technique in 50 cases of partial unilateral deafness. In 30 this was due to Ménière's disease, and in 20 to tumours of or affecting the eighth nerve. Loudness recruitment was found to be present and complete in all 30 cases of Ménière's disease. In 14 of the 20 cases of eighth nerve degeneration it was absent, and in the remaining 6 cases was present but incomplete. Loudness recruitment was thus shown to be characteristically present in a disorder of the cochlear end-organs and to be characteristically absent in a disorder of the cochlear nerve fibres. This finding has since been confirmed by the authors themselves and other workers including Lüscher (1950), Eby & Williams (1951), Kristensen & Jepsen (1952), Lundborg (1952) and Schuknecht & Woellner (1955). Dix & Hood (1953) have themselves pointed out that full loudness recruitment may occur in a small proportion (8%) of cases of eighth nerve tumours, and this they consider attributable to hair-cell changes resulting from interference with the cochlear blood supply. For this explanation, substantial support was derived from their studies of two subjects before and after the removal, effected without further damage to the cochlear nerve, of very small acoustic neurofibromata. The pure tone audiograms and loudness recruitment diagrams obtained before and after the operation in one of these subjects is shown in fig. 3. The pure tone audiograms are substantially the same; loudness recruitment, complete before operation, is absent following it. These results were considered to indicate that loudness recruitment due to interference with the cochlear blood supply can be abolished by its restoration.

This establishment of the anatomical basis of the loudness recruitment phenomenon has proved to be a step of much

FIG. 3. AUDIOGRAMS AND LOUDNESS RECRUITMENT DIAGRAMS OBTAINED IN SUBJECT BEFORE AND AFTER REMOVAL OF SMALL ACOUSTIC NEUROFIBROMA



Left-hand: pre-operative results
Right-hand: post-operative results

A. Audiograms:
Abscissae: frequency (cyc./sec.) (logarithmic scale)
Ordinates: hearing loss (db.)
●——● left ear
x- - - - - - - - -x right ear

B. Loudness recruitment tests
Abscissae: sound intensities for deaf ear (db.)
Ordinates: sound intensities for normal ear (db.)

importance in the further development of the subject. Its clinical implications are clear enough, namely, that it makes possible a differentiation of two types of nerve deafness: nerve-fibre deafness and end-organ deafness. In addition, it has provided a key point for the development of new investigations which have already been of value, not only in bringing about a better understanding of the phenomenon itself, but in providing new data bearing upon a number of more general problems connected with the functional mechanism of the inner ear.

3. Loudness Recruitment and its Relationship to Adaptation

As indicated in the contribution on fatigue and adaptation of hearing in this number of the Bulletin[1], the responses of functionally abnormal cochlear end-organs, as encountered in Ménière's disease, are in certain ways characteristic of those of the fully adapted stretch receptors studied by Matthews (1931). Thus, their initial response to brief intermittent stimuli may be normal or even supranormal. For this, the term " on-effect normality " has been used, and there would seem to be little doubt that it provides, at any rate in part, the physiological basis of the loudness recruitment phenomenon. This normal response, however, cannot be maintained if the stimulus is continued, and there occurs instead a rapid fall, the " relapse phenomenon ", to a subnormal level. The capacity of a normal receptor to maintain its response is dependent upon its capacity for energy restoration, and Hood (1950) has suggested that some failure of this capacity is related to the relapse phenomenon in Ménière's disease. He has explained the essential features of loudness recruitment, i.e., on-effect normality and relapse, in terms of the simple hydrostatic analogy shown in fig. 4. When the taps are opened the initial discharges are of equal height. With the normal receptor the large supply pipe makes possible the maintenance of the discharge at a rather higher level than in the case of the affected receptor with a small supply pipe. The close resemblance, in respect of on-effect normality and the relapse phenomenon, so demonstrated in the responses of Ménière's disease to those of the fully adapted sensory end-organ studied by Matthews is in very good agreement with the collateral anatomical evidence, that loudness recruitment is an expression of end-organ disease. In addition, the constant association with loudness recruitment of the relapse phenomenon is of great practical importance, and cannot be left out of account in the design of test procedures used for the demonstration of the former. It is clear, for instance, that if instead of the short stimuli prescribed for the demonstration of loudness recruitment, long stimuli are used, then the relapse phenomenon will make its appearance and the loudness recruitment phenomenon will be masked.

[1] See Hood, p. 125.—Ed.

This point is well illustrated in fig. 5, which shows the loudness recruitment curves in two subjects with unilateral Ménière's disease. The top curves were obtained by means of the alternate balancing technique, using short interrupted stimuli. Both show full recruitment. Over-recruitment is, in fact, present. The lower two curves show the results in the same subjects when a balancing procedure is used which involves the use of sustained stimuli applied simultaneously to the two ears. The results obtained are shown to be markedly different from the upper curves. In one subject loudness recruitment is abolished and in the other reversed.

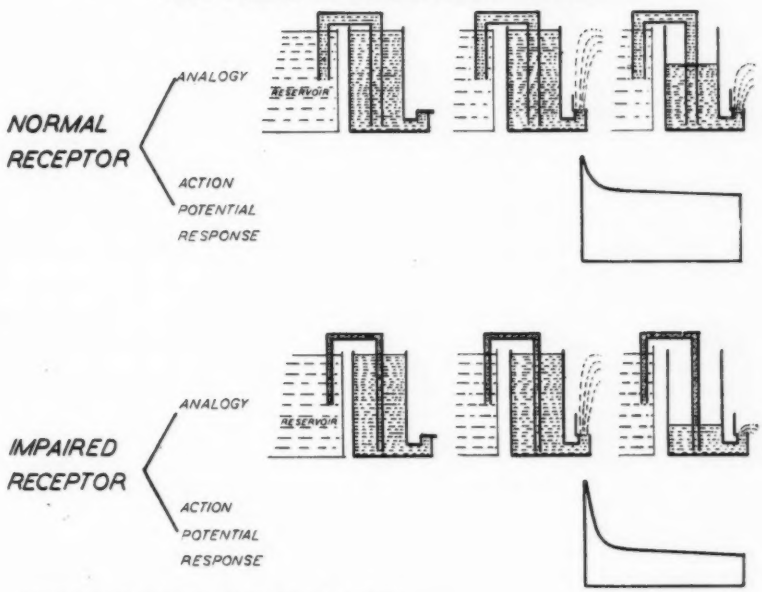
These results are the subjective manifestations of on-effect normality, and of the relapse phenomenon. In the alternate balancing procedure the short tone pulses used give rise to normal on-effects, and to loudness recruitment at high levels of stimulus intensity. In the simultaneous loudness balancing procedure, however, the response of the adapted end-organ is being observed, and the sustained tones used give rise to relapse with a considerable decline in sensitivity resulting in the abolition or reversal of loudness recruitment.

The point is of obvious importance in the design of loudness recruitment tests and will be considered later in greater detail.

4. Loudness Recruitment and Speech Intelligibility

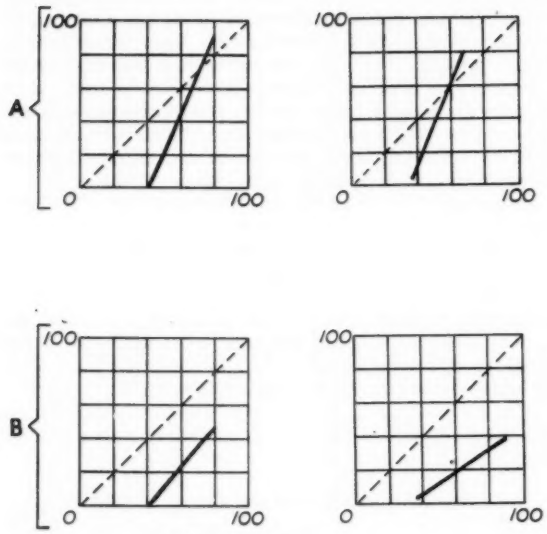
It is well known that the capacity to understand speech tends to be quite differently related to the hearing for pure tones in nerve deafness and conductive deafness. In

FIG. 4. HYDROSTATIC ANALOGY, ILLUSTRATING ESSENTIAL FEATURES OF LOUDNESS RECRUITMENT



Upper set of tanks represents normal receptor.
Lower set represents impaired receptor.
First tank in each set represents quiescent condition.
Second set represents initial responses of receptors to stimulation.
Third set represents adapted responses when energy supplied just balances energy dissipated.

FIG. 5. LOUDNESS RECRUITMENT CURVES IN TWO SUBJECTS WITH UNILATERAL MENIERE'S DISEASE



Left-hand: case 1
Right-hand: case 2
Abscissae: sound intensities for deaf ear (db.)
Ordinates: sound intensities for normal ear (db.)
A: curves obtained with alternate balancing technique
B: curves obtained with simultaneous balancing technique

ness due to a lesion of the conductive mechanism of the middle ear. The deafness for speech is measured by the displacement of the curve to the right of the normal curve at the 40% level for word articulation. The form of the curve shows that with increasing amplification full restoration of speech intelligibility is obtained. The curve B was obtained in a case of deafness due to Ménière's disease. It does not lie parallel to the normal curve, and shows a downward slope towards the right, indicating a disproportionate failure of intelligibility at high intensities.

It would thus appear that loudness recruitment, so typical a feature of end-organ disease, is accompanied by a loss of intelligibility which is characteristically uncompensated by means of amplification.

In deafness due to lesions of the cochlear nerve fibres without loudness recruitment, the restoration of speech intelligibility by means of amplification follows different and more complex lines. Thus, in 1949, Dix, Hallpike & Hood described several cases of deafness due to cochlear nerve degeneration resulting from tumour pressure in which good restoration of intelligibility was obtained with amplification. Similar results have since been reported by Eby & Williams (1951). Subsequent investigations (Dix & Hood, unpublished) have, however, revealed that, in a significant proportion of comparable cases of deafness associated with tumours of the cerebello-pontine angle, restoration of speech intelligibility by means of amplification is not a constant finding.

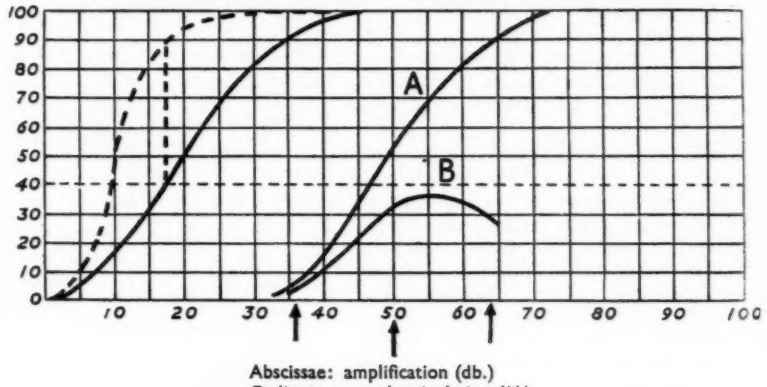
It would thus appear that the achievement of good speech intelligibility with amplification is dependent, not only upon the integrity of Corti's organ as shown by the absence of loudness recruitment, but also upon other factors, of which particular, it is a general clinical experience that in certain varieties of nerve deafness intelligibility of speech cannot be restored by means of amplification, as is the case in conductive deafness.

The correlation, in cases of deafness of known pathology, between the hearing for pure tones and speech intelligibility has now been extensively investigated with particular reference to the significance of loudness recruitment (Dix, Hallpike & Hood, 1949; Eby & Williams, 1951; Huizing & Reyntjes, 1952; and Palva, 1952).

Direct measurement of speech intelligibility is carried out nowadays in accordance with a standardized procedure. The subject is seated with his ear at a fixed distance from a loudspeaker, by means of which lists of words are reproduced. The intensity of the speech sounds reaching the ear can be varied over a wide range, and in this way a direct estimate can be obtained of the patient's ability to understand words and sentences spoken under ordinary conversational and other conditions.

The results are graphically recorded in the manner shown in fig. 6. The curve A was obtained with a subject with deaf-

FIG. 6. DIRECT MEASUREMENT OF SPEECH INTELLIGIBILITY



Abscissae: amplification (db.)
Ordinates: word articulation (%)
Curves for normal subject:
- - - - - - sentences
——— words
A: curve for subject with deafness due to lesion of conductive mechanism of middle ear
B: curve for subject with deafness due to Ménière's disease
Critical level for word articulation: 40 %
Amplification:
36 db.: speech (church level)[2]
50 db.: ordinary conversation at 3 ft.
64 db.: loud conversation

[2] Medical Research Council Special Report Series No. 261, 1947. *Hearing aids and audiometers.* HMSO, London.

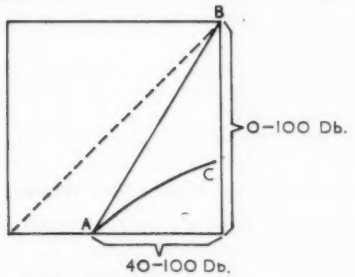
one must be the need for a certain proportion of normally functioning cochlear nerve fibres. In addition, a likely factor is the occurrence in certain cases of eighth nerve tumours of partial loudness recruitment.

5. Technique of Loudness Recruitment Tests with Especial Reference to the Measurement of the Intensity Difference Limen and Pure Tone Audiometry

The alternate loudness balancing technique of Fowler is strictly applicable only to cases of monaural deafness. For the investigation of loudness recruitment in binaural deafness other types of test procedure are, therefore, required and many have been devised. Most of these are dependent upon the demonstration of changes in the normal intensity difference limen. The theoretical relationship between loudness recruitment and the intensity difference limen is shown in fig. 7, adapted from a recent publication by Dix & Hood (1953). This shows a conventional loudness recruitment diagram from a case of unilateral deafness due to Ménière's disease. The deafness at threshold is 40 db., and full recruitment of loudness is obtained at an intensity of 100 db. It follows that the full loudness range, 0–100 db., of the unaffected ear is matched by the much smaller range, 40–100 db., of the affected ear. Loudness recruitment thus involves a compression of the loudness scale, with a resultant diminution of the intensity difference limen. Although the theoretical basis of this equation has recently been called in question by Hirsh (1954), there is little doubt that, in most cases of deafness with loudness recruitment, reduction of the difference limen occurs and can be measured by means of suitably designed test procedures. Various techniques have been described by Lüscher & Zwislocki (1948), Denes & Naunton (1950), Lund-Iverson (1952), Jerger (1952) and others. In all, the stimulus used consisted of a pure tone, varied in respect of intensity. In some cases a continuous modulation was used; in others the stimulus was interrupted. The results obtained have varied. In some instances these have been constant and could be correlated in suitable cases with loudness recruitment as demonstrated by the binaural balancing procedure; in other instances, however, less constant results have been obtained.

It now seems possible, at any rate in part, to explain these variations in the results of the difference limen tests for loudness recruitment in terms of the relapse phenomenon so characteristic of abnormal cochlear sense organs when stimulated in a certain way. Thus, in some of the test procedures described, the intensity changes have been comparatively slow and the intensity level of the stimulus well above the threshold. Under these circumstances, on-effect normality would be minimized and the relapse phenomenon accentuated. As a result, loudness recruitment would be masked and its normal accompaniment, a diminution of the intensity difference limen, abolished. A simple experimental confirmation of this possibility is shown again in fig. 7. In this, a loudness recruitment determination has been made by use of prolonged stimuli instead of the usual short interrupted stimuli. As a result, the loudness recruitment curve is changed from AB to AC: that is to say, the response at high intensities, instead of being recruited, shows a progressive deterioration. Correspondingly, the loudness scale exhibits expansion instead of compression with increase instead of decrease of the difference limen.

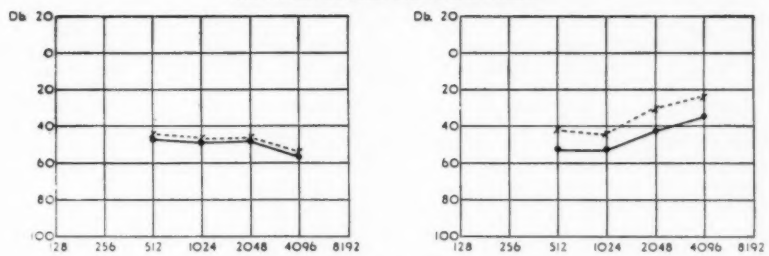
FIG. 7. LOUDNESS RECRUITMENT CURVE IN SUBJECT WITH UNILATERAL DEAFNESS DUE TO MENIERE'S DISEASE



--------- curve for normal ear
AB: curve for affected ear (end-organ deafness), using short stimuli
AC: curve for affected ear, using prolonged stimuli

The same considerations are applicable to the test procedure used for the determination of the pure tone audiometric threshold. Thus, a stimulus of fluctuating intensity is sometimes used, as described by Békésy (1947) for this purpose. Graphic records of the stimulus intensity show a marked diminution of the fluctuation amplitude when loudness recruitment is present, and is diagnostic thereof. This, too, is accepted as evidence of some diminution of the difference limen. Here, again, much depends upon the exact character of the intensity variations. Thus, in fig. 8 are shown the audiograms obtained by a modified Békésy procedure in two cases of deafness. That on the left is due to conductive deafness; that on the right to end-organ deafness. The interrupted-line curves were obtained with a modulator switch, giving a rapid build-up of intensity designed to evoke maximal on-effects. The full-line curves were obtained with a switch giving a much more gradual build-up of intensity. Such stimulation would not evoke maximal on-effects, and would not exclude loss of sensitivity due to adaptation. It will be

FIG. 8. AUDIOGRAMS OBTAINED BY MODIFIED BEKESY PROCEDURE IN TWO CASES OF DEAFNESS



Left-hand audiogram: conductive deafness
Right-hand audiogram: end-organ deafness
Abscissae: frequency (cyc./sec.)
Ordinates: hearing loss (db.)
x- - - - - - - - -x interrupted tone threshold
●————————● continuous tone threshold

noted that the physical form of the stimulus makes little difference to the threshold determination in the case of conductive deafness. In the case of end-organ deafness, however, a considerable rise of threshold results from the use of the slowly rising stimulus.

These findings have an obvious application in the design of pure tone audiometers and make clear that at any rate in cases of end-organ deafness the build-up of the stimulus intensity should be rapid and of standard form.

6. Clinical Value of the Loudness Recruitment Test

The generalization that loudness recruitment is characteristically present and complete in deafness due to disease of the end-organ and absent in cochlear nerve-fibre degeneration has now been confirmed and extended by investigations of a large variety of labyrinthine and retrolabyrinthine disorders, by workers including de Bruine-Altes (1946), Lüscher (1950), Saltzman (1949), Zangemeister (1950), Lundborg (1952) and Kristensen & Jepson (1952). Their results have shown the consistent presence of loudness recruitment in conditions other than Ménière's disease with a clearly defined labyrinthine pathology, such as acoustic trauma, hereditary perceptive deafness, meningitis and syphilis. Absence of loudness recruitment or partial recruitment only, has, on the other hand, been the consistent finding in retrolabyrinthine deafness due to arachnoiditic cysts, affections of the cochlear nerve by mumps and other infective processes, skull deformities including hemiatrophy and platybasia, tabes and brain-stem lesions. A survey of more recent findings (Dix, unpublished) is in full agreement with these results.

These findings are derived from studies of loudness recruitment in cases of unilateral deafness associated with the four following conditions:

Condition	*Number of cases*
i. Virus affections of the eighth nerve	16
ii. Disseminated sclerosis	13
iii. Acoustic trauma	12
iv. Otitic labyrinthitis	7

In all of these conditions reliable data are available concerning the site and nature of the lesion responsible for the deafness. Thus, in virus affections Denny-Brown, Adams & Fitzgerald (1944) have described the occurrence of widespread retrolabyrinthine haemorrhagic and inflammatory changes in the sensory ganglia, the meninges, the extra-medullary nerve fibres and finally the medulla itself.

In disseminated sclerosis the deafness is due to demyelinating lesions of the cochlear nervous connexions within the brain-stem. The results of loudness balance tests in these two groups of nerve-fibre deafness showed that in 13 of the 16 cases of virus affection, and in 10 of the 13 cases of disseminated sclerosis, loudness recruitment was either absent or incomplete.

In our third group, due to acoustic trauma, the lesion is well known to affect the hair cells of Corti's organ. Group 4 consisted of cases with middle-ear infections, with secondary otitis interna as shown by the occurrence of vertigo and exacerbation of deafness. In none was there neurological evidence of extension of infection beyond the labyrinth. Loudness balance tests in these two groups of end-organ deafness showed that in 11 of the 12 cases of acoustic trauma, and in 6 of the 7 cases of otitic labyrinthitis, loudness recruitment was present and complete.

ACKNOWLEDGEMENTS

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FATIGUE AND ADAPTATION OF HEARING

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1 Fatigue
  a Variation with stimulus duration
  b Variation with stimulus intensity
  c Recovery
2 Adaptation
  a Variation with stimulus duration
  b Recovery
  c Variation with stimulus intensity
3 Pathological adaptation
References

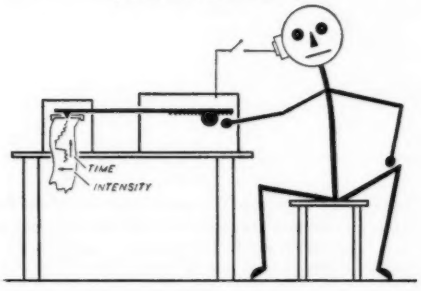
Pure tone stimulation brings about certain reversible derangements of cochlear function. These fall into two categories, with characteristics in many ways similar to those of sensory fatigue and adaptation, as defined in accordance with accepted physiological usage, and will be considered separately under these two headings.

1. Fatigue

Our knowledge of the physiological features of fatigue is derived principally from the investigations of Bronk (1929) of the action potential response of single stretch receptors of voluntary muscle to stretch stimuli. Bronk found that fatigue was increased by nitrogen, retarded by oxygen, and uninfluenced by changes of ionic concentration in the media surrounding his preparations. One characteristic of fatigue is its persistence after the removal of the stimulus. For this reason its subjective counterpart in the human subject, often termed post-stimulatory fatigue, is conveniently studied in the manner shown in fig. 1.

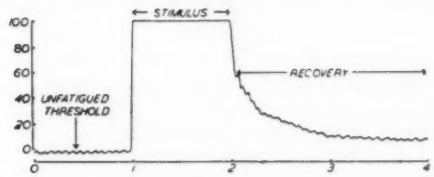
The attenuator of the audiometer on the left is connected by a rack and pinion to a writing mechanism whereby variations of intensity are continuously recorded. Initially the subject traces his auditory threshold by varying the attenuation in a rhythmical manner, so that the peaks represent the "just heard" points. The intensity of the tone is then raised 100 db. above this level and maintained for one minute, after which the subject again traces his threshold. Its elevation gives a measure of the fatigue, and it will be seen in the example shown in fig. 2 that recovery is slow and incomplete one minute after removal of the fatiguing tone.

FIG. 1. METHOD OF STUDYING AUDITORY FATIGUE
(See text, section 1)



An obvious point of difference between this procedure and that used in the physiological studies of Bronk deserves attention. Bronk's observations were, at all stages, confined to the responses of one or a few receptors, an arrangement very different from that in the human experiment described. Thus it is generally agreed that, with increase of pure tone intensity, there occurs firstly an increase of the stimulus intensity affecting that small group of receptors, termed for convenience the central group, which is concerned with perception at normal threshold intensity. In addition, the stimulus spreads beyond these to involve other receptors termed, again for convenience, the peripheral group. Thus, following the application of a fatiguing stimulus of high intensity, there will be a central group of receptors in which fatigue is high, and a peripheral group in which fatigue is lower. The extent of this peripheral group and the contour of the fatigue distribution between it and the central group will be dependent upon the intensity and duration of the fatiguing stimulus. It follows that the physiological basis of the threshold elevation resulting from a fatiguing pure tone stimulus is a complex one. Nevertheless this elevation has been usefully employed as an empirical measure of fatigue and to evaluate its dependence upon the duration and intensity of the fatiguing stimulus.

FIG. 2. AUDITORY FATIGUE: THRESHOLD ELEVATION FOLLOWING A FATIGUING STIMULUS OF 100 db. SENSATION LEVEL



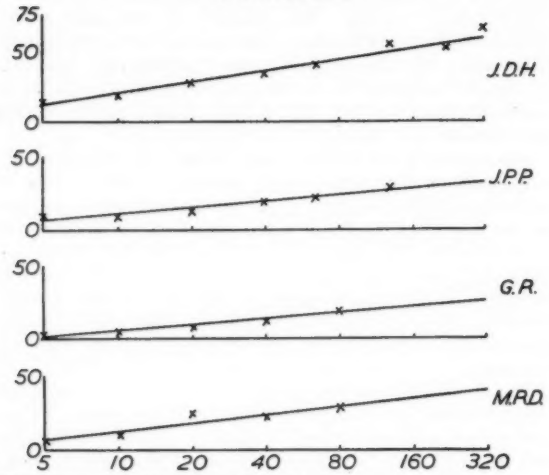
Abscissae: time (min.)
Ordinates: intensity above threshold (db.)
Frequency: 1,000 cyc./sec.

a. Variation with Stimulus Duration

Using the test procedures described, the stimulus intensity was maintained constant at 100 db. sensation level, and the fatigue measured after stimulus durations up to 320 seconds. Each observation was made 10 seconds after removal of the fatiguing stimulus, and the results for four subjects are shown in fig. 3.

It will be observed that, as in the case of the end-organ fatigue observed by Bronk, auditory fatigue continues to increase as the stimulus duration increases. Similar results have been reported by Davis and his co-workers using higher stimulus intensities and much longer durations (Davis, Morgan, Hawkins, Galambos & Smith, 1950).

FIG. 3. VARIATION OF FATIGUE WITH DURATION OF STIMULUS

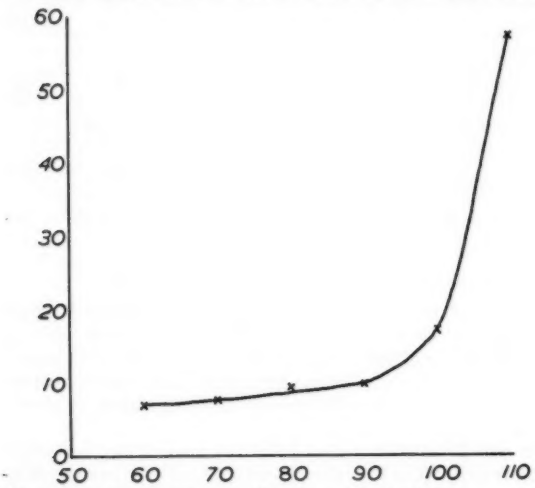


Abscissae: duration of stimulus (sec.)
Ordinates: threshold elevation 10 seconds after cessation of stimulus (db.)
Stimulus frequency: 2,000 cyc./sec.
Stimulus intensity: 100 db. above threshold

b. Variation with Stimulus Intensity

Bronk found that the rate of decline of the action potential response was a function of stimulus intensity, a finding which many authors have now substantiated in respect of auditory fatigue. A typical set of results is given in the curve of fig. 4, which shows the elevation of the threshold 10 seconds after the cessation of a fatiguing tone of 2,000 cyc./sec. of one-minute duration, with sensation levels from 50 to 110 db. As will be seen, fatigue increases progressively with stimulus intensity. The inflexion of the curve in the region 90–100 db. suggests a dividing line between two different kinds of end-organ changes—those that are physiological and reversible, and those that are pathological and irreversible.

FIG. 4. AUDITORY FATIGUE: VARIATION OF THRESHOLD ELEVATION WITH STIMULUS INTENSITY



Abscissae: stimulus intensity (db. above threshold)
Ordinates: threshold elevation 10 seconds after cessation of stimulus (db.)
Frequency: 2,000 cyc./sec.

c. Recovery

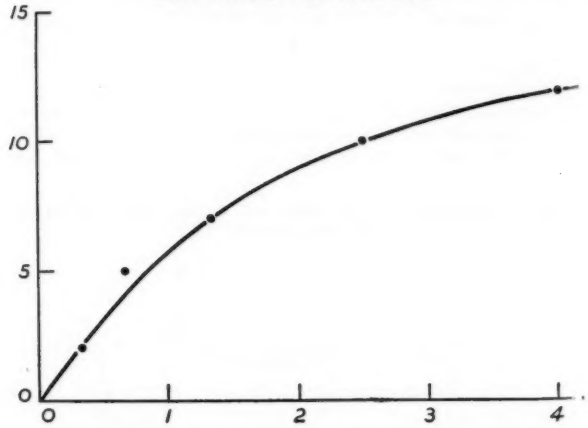
Bronk's findings indicate clearly that the rate and total duration of the recovery process are functions of the magnitude of the initial fatigue. Parallel results have been obtained with auditory fatigue, and in fig. 5 is shown the recovery plotted against the magnitude of the fatigue measured 10 seconds after the cessation of fatiguing tones of one-minute duration and varying intensity. This shows that, as fatigue increases, the recovery period becomes longer.

2. Adaptation

Adaptation may be described as a process of equilibration whereby the energy which is expended in the response is just balanced by the energy which becomes available to maintain it. The studies of Matthews (1931) of the action potential response of single muscle stretch receptors have provided useful criteria in distinguishing between fatigue and adaptation. In contrast to fatigue, adaptation is little influenced by the concentration of free oxygen or nitrogen in the surrounding media; it is, however, very sensitive to the action of certain ions.

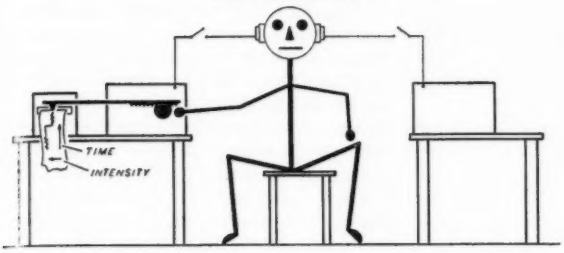
It is transient in nature and characterized by a decrement in the response which occurs during the course of application of the stimulus, and for this reason the technique shown in fig. 6 is best suited to the investigation of auditory adaptation (Hood, 1950). A tone of fixed intensity, say 80 db., is applied to the subject's left ear (the test ear), while simultaneously a tone of the same frequency, say 1,000 cyc./sec., from another audiometer is applied to the right ear (the control ear), and

FIG. 5. VARIATION OF RATE OF RECOVERY WITH MAGNITUDE OF FATIGUE



Abscissae: time of recovery to pre-fatigue threshold (min.)
Ordinates: threshold elevation 10 seconds after cessation of stimulus (db.)
Frequency: 2,000 cyc./sec.

FIG. 6. METHOD OF STUDYING AUDITORY ADAPTATION



its intensity varied to give equality of loudness. This balance is obtained with an intensity of 80 db., as will be seen from the tracing shown in fig. 7. The tone in the control ear is then removed for a period of three minutes in the example shown, while that in the test ear is continued. A further loudness balance is then made, and it will be seen that equality of loudness is now obtained with an intensity of only 50 db. in the control ear.

The loss of 30 db. is the measure of the sensitivity loss due to adaptation sustained in the intervening three minutes by the test ear. This constitutes, in its simplest terms, the measurement of auditory adaptation.

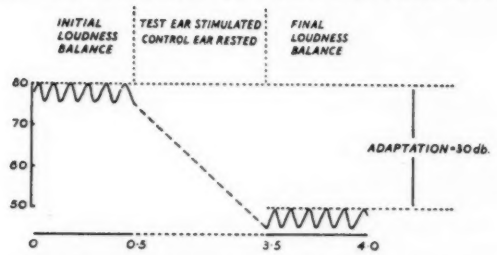
It will be observed that, in contrast to the conditions of the test used for the measurement of auditory fatigue, the test stimulus is maintained during the measurement of the sensitivity loss. This, therefore, provides a measure of the functional alterations occurring in a particular group of receptors during the actual process of their response to the test stimulus.

a. Variation with Stimulus Duration

In fig. 8a is shown the action potential response recorded in the auditory nerve of the cat by Derbyshire & Davis (1935) to a 1,500 cyc./sec. tone. It is characterized by an initial high-frequency discharge, the well-known " on-effect ", which declines rapidly to a low level and is followed by a much slower decline; this, unlike the decline in fatigue, progresses for some 3 or 4 minutes and thereafter remains constant. Derbyshire and Davis refer to these two rates of decline as fast and slow equilibration respectively.

These results have their counterpart in the investigation of auditory adaptation exemplified in fig. 9. As before, a tone of fixed intensity, in this case 1,000 cyc./sec. at 80 db., was

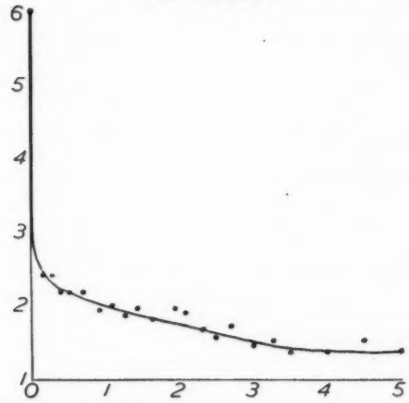
FIG. 7. MEASUREMENT OF AUDITORY ADAPTATION



Abscissae: time (min.)
Ordinates: intensity above threshold (db.)
Stimulus intensity: 80 db.
Stimulus frequency: 1,000 cyc./sec.

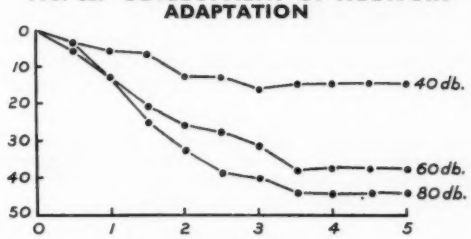
applied to the test ear; and the tracing shown is taken, as before, from the control ear. It differs from the tracing of fig. 7 only in that a simultaneous balance was made repeatedly over periods of 10 seconds at intervals of 20 seconds, during which time the tone in the control ear was removed. The tone in the test ear was continued throughout the duration of the test. It will be seen that adaptation, as measured by the fall in the balancing intensity, reaches its maximum, 35 db., in approximately three or four minutes, and thereafter remains constant. The development of adaptation, using three different intensity levels of the test tone at 1,000 cyc./sec., is shown in fig. 8b. With all three, adaptation reaches a maximal level at about the same time independently of the intensity of the test tone.

FIG. 8a. EQUILIBRATION IN THE AUDITORY NERVE OF THE CAT



Abscissae: time (min.)
Ordinates: amplitude of action potential response (mm.)
Stimulus frequency: 1,500 cyc./sec.

FIG. 8b. DEVELOPMENT OF AUDITORY ADAPTATION



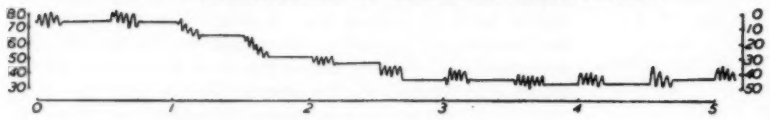
Abscissae: time (min.)
Ordinates: adaptation (db.)
Stimulus frequency: 1,000 cyc./sec.

A comparison with fig. 8a will show that the time-courses of the development of adaptation, as manifested respectively by decline of the action potential responses and of loudness, are in close accord, so supporting the view that they are manifestations of the same phenomenon.

b. Recovery

The fast and slow equilibration of Derbyshire and Davis occurs also in the results of Matthews, reproduced schematically in fig. 10; he further studied the recovery processes.

FIG. 9. DEVELOPMENT OF AUDITORY ADAPTATION



Abscissae: time (min.)
Ordinates:
Left-hand: intensity above threshold (db.)
Right-hand: adaptation (db.)
Stimulus frequency: 1,000 cyc./sec.
Stimulus intensity: 80 db. above threshold

These fall into two parts: (i) the recovery of the on-effect and (ii) the recovery of slow equilibration. The recovery of the on-effect is rapid. If, following adaptation, the stimulus is removed and then re-applied after a rest period of less than one second, the on-effect is present but diminished in magnitude.

If the rest period is greater than one second, the on-effect is completely restored to normal, but continued stimulation results in a rapid relapse to a level which is determined by the duration of the rest period. Matthews refers to this relapse as the " adaptation remainder ". Thus the capacity to respond initially at a high rate returns much more rapidly than the capacity to maintain the discharge at a high rate. Matthews investigated the latter by plotting the number of impulses in the first five seconds of re-stimulation following rest periods of varying durations. His results are shown in fig. 11a, and it will be seen that recovery of the response to sustained stimulation is much slower than the on-effect, and is complete only after some 30 seconds or more.

Matthews' results, in respect of the recovery of the on-effect, find their counterpart in the investigations of Lüscher & Zwislocki (1949), who used a stimulating tone of 80 db. intensity at 3,000 cyc./sec. with a duration of from 0.04 seconds' to 2 seconds' duration, and followed this, after a brief rest interval of from 0.01 seconds to 1 second, with a test tone of the same frequency. The effect of the stimulating tone was measured as an elevation of the threshold of the test tone above its unadapted value. With very short rest intervals these workers found that the threshold of the test tone was raised by as much as 40 db., but recovery was nearly complete within 0.2 seconds. Similar results have been obtained by de Maré (1939), Gardner (1947) and others, in good agreement with those of Matthews.

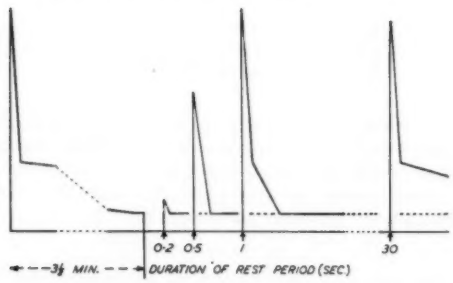
In fig. 12 is shown the experimental procedure used in investigating the recovery of the cochlea from those effects of sustained stimulation that may be taken as the subjective counterpart of slow equilibration. As before, the first operation was to make the usual loudness balance, in the case shown, at 80 db. at 1,000 cyc./sec. The control ear was then rested while the test ear was stimulated for at least three minutes. At the end of this period the adaptation would normally have reached the level shown by the lowest tracing, that is, 30 db. At this point, however, both ears were rested for periods varying from a few seconds to two minutes. At the end of this rest period the stimulus tone was applied at 80 db. as before to the test ear, while simultaneously the variable tone was re-applied to the control ear, with resumption of the balancing procedure.

In fig. 12 are shown the different stages of recovery after four different rest periods—0 seconds, 10 seconds, 30 seconds and 60 seconds. In this way it was possible to show that the process of recovery from adaptation takes the form of the curve shown in fig. 11b. A comparison of this with that of fig. 11a showing the recovery of slow equilibration shows a general resemblance of the time-courses of the two processes; this suggests a significant relationship between the phenomena observed by Matthews in the peripheral nervous system and the neural mechanism which underlies the subjective phenomena observed by ourselves.

c. Variation with Stimulus Intensity

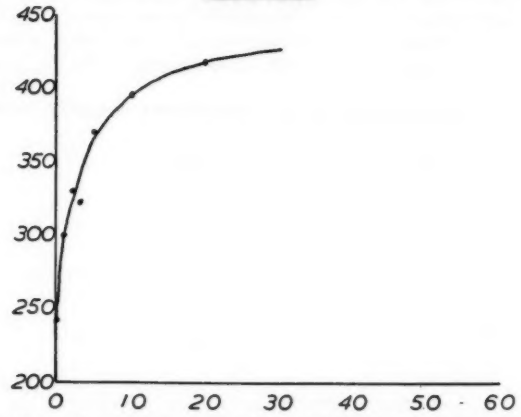
It is well known that the response rate of sensory receptors increases progressively with stimulus intensity, and here the relevant studies of Galambos & Davis (1943) of single-fibre preparations of the auditory nerve are of particular interest. Their main conclusions were that, as the stimulus intensity is raised, not only does the rate of response of individual nerve fibres increase, but more and more fibres are brought

FIG. 10. RECOVERY OF ACTION POTENTIAL RESPONSES FROM MUSCLE STRETCH RECEPTORS FOLLOWING RE-APPLICATION OF STIMULUS AFTER VARYING REST PERIODS



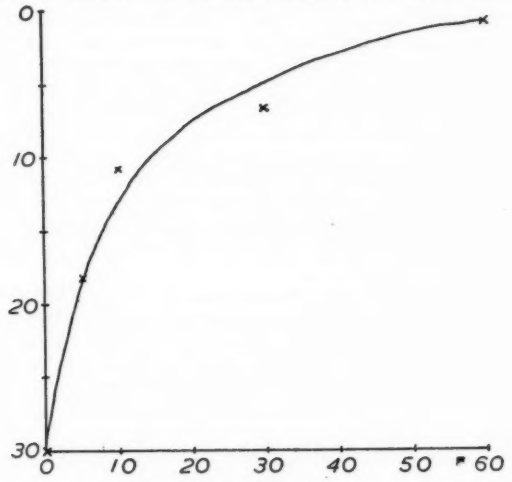
Abscissae: time
Ordinates: action potential response

FIG. 11a. ADAPTATION IN A SINGLE END-ORGAN: RECOVERY



Abscissae: duration of rest period (sec.) following loading period of 10 seconds
Ordinates: number of impulses/5-sec. period

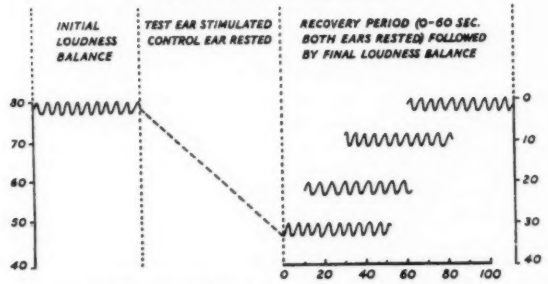
FIG. 11b. RECOVERY FROM AUDITORY ADAPTATION



Abscissae: time (sec.)
Ordinates: adaptation (db.)
Stimulus intensity: 80 db.
Stimulus frequency: 1,000 cyc./sec.

into activity. They write that each nerve fibre has a characteristic frequency and intensity. In other words, the frequency which first excites " . . . is the most effective at any intensity, yet as the intensity level is raised, activity is caused by frequencies at some distance from (and particularly those below) the characteristic frequency". They further studied the effect of an intensity increase upon the fully adapted nerve fibre and found that this was accompanied by an increase in the discharge rate which eventually reached a maximum beyond which a further increase in intensity produced no further increase in the frequency of the response. The maximal discharge rate was, of course, well below the unadapted maximum. One nerve fibre, in fact, maintained a constant adapted discharge rate despite an intensity increase of 50 db.

FIG. 12. RECOVERY FROM ADAPTATION AFTER VARYING REST PERIODS



Abscissae: time (sec.)
Ordinates:
Left-hand: intensity above threshold (db.)
Right-hand: adaptation (db.)
Stimulus intensity: 80 db.
Stimulus frequency: 1,000 cyc./sec.

These results accord well with our conception of adaptation. The maximal rate of response of the end-organ is determined by its relative refractory period. Once this maximal rate has been achieved, any further increase in stimulus intensity can invoke no further increase in response.

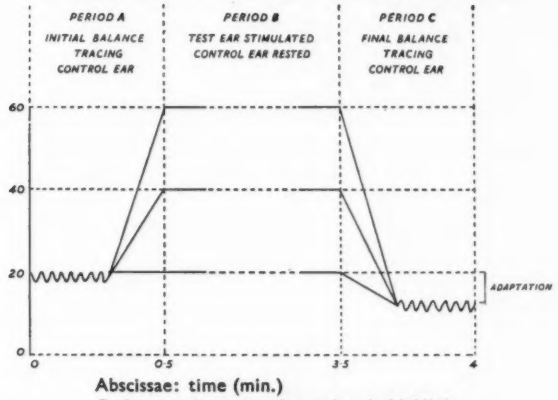
Now, since adaptation represents the attainment of a state of dynamic equilibrium between the energy expended in the response and the energy which becomes available to maintain it, a condition characteristic in general of the all-or-none type of response, it can be said to result solely from the activity of the end-organ. It is, in other words, dependent only upon the rate of response of the end-organ, and, when the rate of response is maximal, the magnitude of the adaptation is maximal and no further increase in stimulus intensity will induce further adaptation. We come, therefore, to the important conclusion that, within the limits outlined above, adaptation in the individual receptor is independent of the intensity of the stimulus.

It will be seen from fig. 8b that, as the stimulus intensity is raised from 40 db. to 60 db. and to 80 db., the maximal fall in the response increases from 10 db. to 35 db. to 45 db. In this respect, therefore, the amount of adaptation varies directly as the stimulus intensity. It is clear, however, that the receptor group activated by the 60 db. stimulus will be numerically greater than that activated by the 40 db. stimulus and similarly with the 80 db. and 60 db. stimuli. These, therefore, are receptor groups of different sizes.

It is of interest now to examine the effect of stimuli of varying intensities upon a specific receptor group. The test procedure for this purpose will best be understood from the tracings of fig. 13. This may be explained as follows: initial loudness balances were carried out with a stimulus intensity at the test ear of 20 db. Following this, the test ear was stimulated for three minutes with intensities from 20 db. to 60 db. This was followed by the final loudness balance with the control ear, the intensity at the test ear reverting to its original level of 20 db.

It will be seen that under these conditions we begin (period A) by examining the behaviour of a group of nerve elements in the test ear of specific size, namely those responding to a stimulus of 20 db. Following period A, the stimulus intensity

FIG. 13. EFFECT OF STIMULI OF VARYING INTENSITIES UPON A SPECIFIC RECEPTOR GROUP



Abscissae: time (min.)
Ordinates: intensity above threshold (db.)
Stimulus frequency: 1,000 cyc./sec.

is increased in successive tests by 20 db. up to a maximum of 60 db. (period B), during which time we may suppose the receptors with which we are concerned at the 20 db. level are subjected to much higher stimulus intensities, while in addition other receptors beyond this group are excited. In period C the test tone reverts to 20 db., and the final balance is made.

The general character of the results indicates that the adaptation occurring in the group of receptors of finite size that is excited by the 20 db. stimulus is constant irrespective of the intensity of the stimulus applied thereto. It is thus clear that the adaptation produced in a given group of receptors by a stimulus is the same, whether it be produced by a stimulus which is just sufficient for their physiological activation, or by a stimulus of greater intensity whereby they are subjected to considerably greater physical forces. The fall in the response to these receptors is, therefore, a function solely of their physiological activity, the extent of which is limited by the all-or-none law governing neural activity. According to this, the nature of the response is such that a stimulus either excites or fails to excite the neural elements, and the magnitude of its response is not, therefore, a function of the stimulus intensity.

On this basis, we can regard the neural elements concerned in the small group of receptors reacting to a 20 db. stimulus as reacting maximally. With a 60 db. stimulus, the stimulus intensity reaching the receptors is increased, but their response and therefore their adaptation increases not at all.

Post-stimulatory fatigue, on the other hand, does increase with the stimulus intensity, so that the two varieties of loss of cochlear sensitivity that we have described have this important physiological distinction: the one is a diminution of hearing acuity caused by the physical stimulus and varying in magnitude with its intensity, and the other, adaptation, is a decline in sensitivity of the hearing receptors consequent upon their physiological activity.

3. Pathological Adaptation

Studies of experimental disturbance of end-organ and nerve-fibre function through oxygen deprivation and otherwise (Matthews, 1931; Kugelberg, 1944) suggest that, while adaptation may be markedly accentuated, the excitability to brief intermittent stimulation may be normal or even increased. In other words, the on-effect may be of more than normal magnitude, but sustained stimulation results in a pathological relapse of the response to a subnormal adapted level. In practice these two aspects of disordered end-organ function are strikingly exemplified in Ménière's disease in which structural changes occur in the hair cells of Corti's organ.

Investigations of the kind described in the preceding paragraphs, upon subjects with unilateral deafness due to Ménière's disease, have shown that the affected ear exhibits an abnormal degree of adaptation, a finding which has proved of value in devising test procedures for the differential diagnosis of deafness due to disease of the end-organ and of the conductive mechanism (Hood, 1955).

Loudness recruitment, the other invariable accompaniment of end-organ disease, manifests itself only with tones of short duration, and it would seem possible to explain this phenomenon in part, at any rate, in terms of the increased excitability of the end-organ to brief intermittent stimuli.

These two inseparable accompaniments of end-organ disease call for special attention in the design of all clinical audiometric tests, and will be given further consideration in the paper on loudness recruitment, in this number of the Bulletin.[1]

[1] See Dix, p. 119 of this number of the Bulletin.—Ed.

ACKNOWLEDGEMENT

We acknowledge with thanks the permission of the editor of *Acta oto-laryngologica, Stockholm,* to reproduce figs. 1–4 and 6–12, from Supplement No. 92 to that journal (Hood, 1950).

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THE INVESTIGATION OF VESTIBULAR FUNCTION

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A Introduction
B Caloric tests
C Rotational tests
D Positional tests of otolith function
E Tests for optokinetic nystagmus
References

A. INTRODUCTION

The neuro-otologist's approach to the vestibular system is based upon a concept of its structure and function founded on facts established by Retzius, Ewald and others, and modified by clinical observation.

According to this concept the vestibular system, though normally stimulated and responding as a whole, is organized in two halves, right and left, anatomically distinct and with functions which are separate but complementary. For each of these halves is provided a set of peripheral sense organs, a group of nuclei in the brain-stem and, above this, certain higher centres. These lie in the cerebral hemispheres where they appear to be localized in the posterior parts of the temporal lobes, and may be presumed to subserve the perception of vestibular sensation. In addition, they would seem to exercise some modifying action upon the reflex motor activities of the vestibular nuclei. Beyond this, the vestibular nuclei are connected to the lower motor centres in the brain-stem and spinal cord. The main function of the vestibular system, in which it is assisted by the cerebellum, is executed silently through these motor connexions and consists of a continuous adjustment of the body musculature in response to accelerations including that due to gravity. It thus regulates both movement and posture. Perception of vestibular activity occurs rarely, and then only under conditions of disease or abnormal stimulation, such as occur very notably in the course of certain clinical tests of vestibular function.

Three principles are commonly used in explaining the working of the system. First is the principle of vestibular tonus. At rest, the whole system is considered to be in a state of tonic activity manifested by a steady discharge of action potentials, particularly in its peripheral neurones.

The second principle is that of tonus imbalance. The tonic actions of the two halves of the system upon the eyes and skeletal musculature are normally equal and opposite, and upon this balance normal equilibrium is dependent. Should the tonic action of one half be reduced, then the normal action of its opposite will predominate and bring about activity of the muscles under its control. One striking result of such imbalance is ocular nystagmus. For example, one of the tonus effects of the left half of the system is considered to be the maintenance of an ocular nystagmus with its rapid component to the left. A left vestibular lesion will reduce or abolish this effect, and the result will be a tendency to—or actual—nystagmus to the right, brought about by the preponderant tonus effect of the right half of the system.

The third principle is concerned with the coding of the signals from the receptor organs for angular acceleration and gravity within the labyrinth. Here, the background for the signals is provided by the so-called resting discharge of the vestibular neurones. Turning movements of the head, or changes of its position, bring about fluctuations of this discharge, and are signalled thereby. The arrangement suggested by Hallpike & Hood (1953) for a semicircular canal is shown in fig. 1.

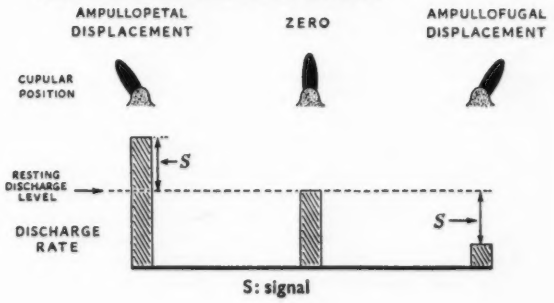
Manifestations of vestibular dysfunction as revealed by investigation are of two kinds, spontaneous and induced. The spontaneous manifestations consist of ocular nystagmus, ataxia of gait, and other abnormalities of muscular control. All come within the category of imbalance phenomena.

Nystagmus is of particular importance. When due to damage of the end-organs, or of the peripheral vestibular neurones, its course is cut short by central processes of compensation, and seldom exceeds a few weeks. When prolonged beyond this, it is usually the result of a lesion of the central vestibular elements.

The induced manifestations chiefly consist of abnormalities of the reflex responses, in particular ocular nystagmus, to stimulation of the vestibular end-organs, and it is the constant endeavour of otologists and others concerned with the design of these test procedures to specify, as clearly as possible, both the physical characteristics of the stimuli and the end-organ or organs stimulated.

Abnormal responses are commonly taken to indicate disease of the end-organs stimulated, or of their associated peripheral nervous pathways. Here some recasting of our ideas is necessary. Thus the responses, being mediated by the vestibular nuclei, are dependent to an important extent upon the latter's functional state. This state, though greatly influenced by the resting discharges from the associated

FIG. 1. FLUCTUATION IN RESTING DISCHARGE LEVEL IN HORIZONTAL SEMICIRCULAR CANAL WITH CUPULAR DISPLACEMENT



labyrinths, is not entirely dependent thereon, and increasing emphasis is at present being laid upon the part played by other elements of the central nervous system, in particular the posterior parts of the temporal lobes, in regulating the activity of the vestibular nuclei. From this it follows that the clinical significance of abnormalities of the vestibular test responses can no longer be limited to the labyrinth and peripheral vestibular neurones and that the neuro-otologist must prepare to extend the diagnostic scope of his tests to include the various vestibular elements within the central nervous system, disorders of which constitute in man so important a cause of vestibular dysfunction.

The spontaneous manifestations of vestibular dysfunction, though of great clinical importance, will not be directly considered in the course of the present contribution. Instead, attention will be devoted to certain of the induced manifestations for which we are dependent upon the following test procedures:

i. Rotational tests and caloric tests of semicircular canal function.
ii. Positional tests of otolith function.
iii. Tests for optokinetic nystagmus.

For the introduction of these tests we are indebted, for the most part, to Bárány. For his work upon them he was awarded the Nobel Prize in Medicine in 1914, and they have since come to constitute the chief stand-by of all who are called upon to undertake the objective examination of vestibular function.

B. CALORIC TESTS

The peripheral mechanism of caloric stimulation was simply explained in 1906 and 1907 by Bárány upon the basis of convection currents in the endolymph of the external semicircular canal. This explanation he founded upon a number of simple observations whereby the induced effects, in particular ocular nystagmus, were shown to exhibit a certain systematic relationship to the temperature of the stimulus and to the spatial orientation of the canal.

Bárány's views have since found abundant support from the very large amount of experimental and clinical evidence which has subsequently been made available. Thus, Schmalz (1932), using purely mathematical methods, was able to provide a detailed reconstruction of the physical events which occur in the canal in response to caloric and rotational stimulation, while direct observations of the cupular movements which occur in response to caloric stimulation were described in 1931 by Steinhausen. Dohlman (1925) observed the magnitude and time-course of the temperature changes induced by caloric stimulation in the canal of the cadaver, and similar studies in the living human subject were reported in 1954 by Cawthorne & Cobb. As a result of this and many other investigations, Bárány's concept may be said to have maintained its position without serious challenge.

Development of the tests has followed two main paths: quantitative and qualitative. Two widely differing schools of thought have regulated their quantitative use. Thus, by many, the test has been employed as an all-or-none indication of vestibular function; a very strong stimulus being used (ice-water) and a negative response accepted as evidence of a dead labyrinth. Kobrak (1918), on the other hand, used short thermally graded stimuli to estimate the nystagmus threshold, and attempted in this way the quantitative evaluation of much smaller sensitivity changes. Qualitatively, the tests had at first an uncomplicated purpose, namely, the measurement of labyrinthine sensitivity. The later work, however, of de Barenne & de Kleyn (1923), Barré (1937, 1938) and others made it evident that the responses were dependent, not only upon this but also upon another factor termed by de Barenne and de Kleyn " Nystagmusbereitschaft ". The effect of this factor was that ocular nystagmus induced in a particular direction was increased; that in the opposite direction diminished. The phenomenon, to which the better descriptive title of directional preponderance has since been applied, is frequently brought about by partial unilateral lesions of the labyrinth or eighth nerve, such as Ménière's disease or tumours of the eighth nerve. It may also be a prominent sign of unilateral lesions of the vestibular nuclei, brought about, for example, by an occlusive lesion of the posterior inferior cerebellar artery.

In addition, as lately demonstrated by Fitzgerald & Hallpike (1942), and by Carmichael, Dix & Hallpike (1954), it may be dependent upon a lesion of the posterior part of the temporal lobe towards which the directional preponderance is directed. The phenomenon has thus acquired a high degree of clinical importance.

In 1942, Fitzgerald & Hallpike described a new caloric test procedure designed to fulfil the following requirements:

i. that the tests should be easy to apply, and comfortable for the subject;
ii. that the results should provide an accurate and repeatable measure of the sensitivity of the external canal;
iii. that they should be capable of revealing the phenomenon of directional preponderance.

The technique has since been in routine use at the National Hospital, Queen Square, London, and will be briefly described. Thereafter a description will be given of the results obtained, their interpretation, and finally, their application to certain common problems of diagnostic neuro-otology.

The test procedure is shown in fig. 2. The head position brings the external canal into the vertical plane, in which its sensitivity is maximal. This, and the use of stimuli of moderate intensity, limit their effect to the external canal. The temperatures used, 30° C. and 44° C., are comfortable, and, being equidistant from body temperature, are calculated

FIG. 2. CALORIC TEST PROCEDURE

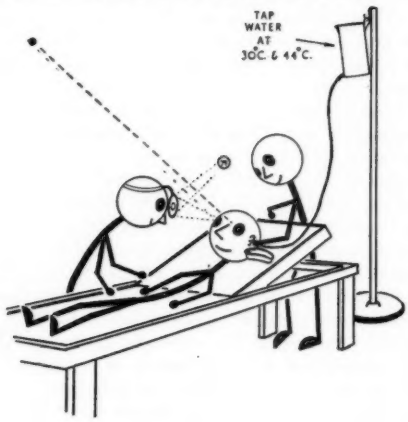
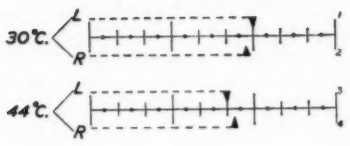


FIG. 3. CALORIC TEST RESULTS OBTAINED IN AN AVERAGE NORMAL SUBJECT



NORMAL

Continuous lines: time (3 min. divided into intervals of 10 sec.)
Dotted lines: response durations

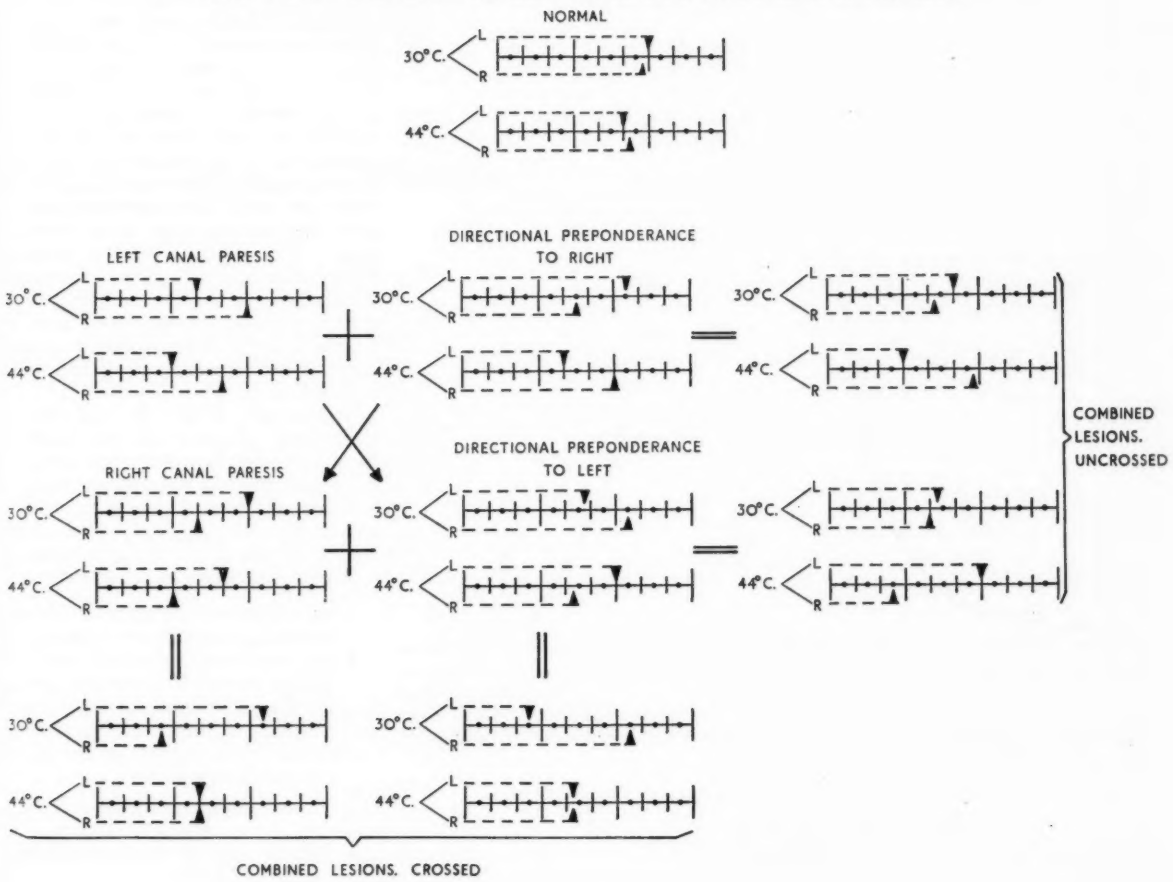
to bring about equal and opposite cupular deflections. The stimulus duration is 40 seconds, during which period not less than 250 ml. of water should flow through the external auditory meatus. The effects are measured in terms of the time interval between the application of the stimulus and the end of the nystagmic response. The observations are carried out in the straight-ahead position of gaze, and are thus of the so-called second degree nystagmus.

The method of recording the results is shown in fig. 3. The diagram represents the results obtained in an average normal subject. The continuous lines represent time: 3 minutes divided into intervals of 10 seconds. The dotted lines represent the response durations. The reactions are conveniently numbered 1 to 4 from above. Reactions 1 and 4 consist of nystagmus with its rapid component to the right; reactions 2 and 3 of nystagmus to the left. Apart from total sensitivity, represented by the sum $(1 + 2 + 3 + 4)$ of the four reaction times, the two important deviations from the normal pattern are:

i. Left/right sensitivity difference, or so-called canal paresis. This is represented by the formula $(1 + 3) - (2 + 4)$.
ii. Directional preponderance. This is represented by the formula $(1 + 4) - (2 + 3)$.

The four primary deviations, left and right canal paresis and directional preponderance to the left and right, are shown in fig. 4. With lesions of the eighth nerve or labyrinth, canal paresis is often associated with directional preponderance to the opposite side. The cause of this is still obscure, although Cawthorne, Fitzgerald & Hallpike (1942) have suggested that it is due to a loss of tonic impulses originating in the otolith organs, in particular the utricle. Whatever its cause, its occurrence as a manifestation of organic disorders of the

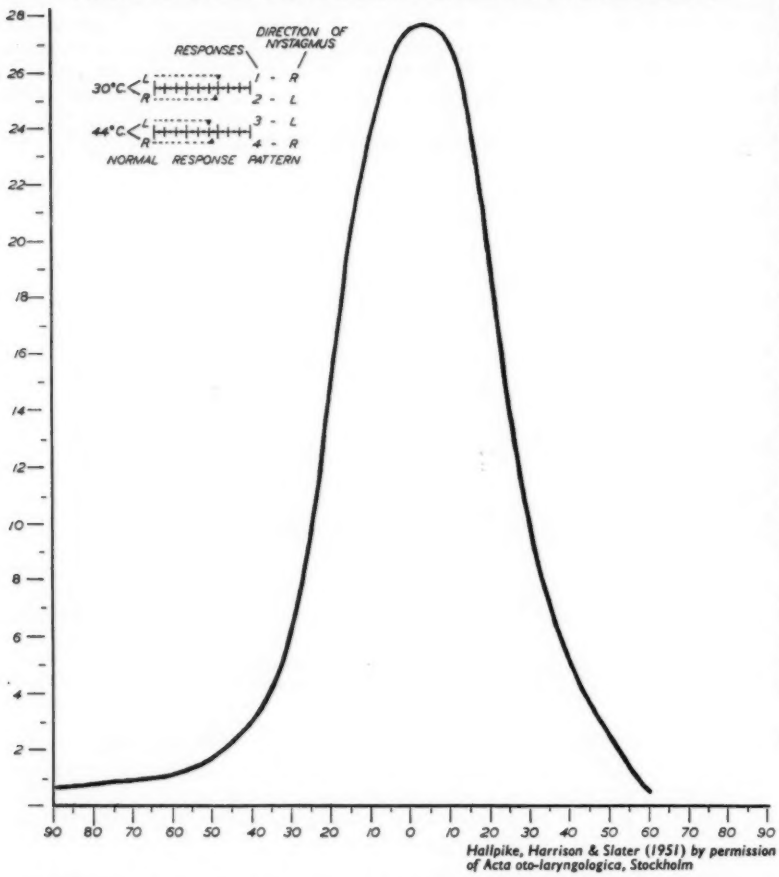
FIG. 4. FULL RANGE OF ABNORMALITIES OF CALORIC TEST RESULTS



labyrinth or eighth nerve is of high significance. It may occur alone, or in combination with a canal paresis. Such combinations are represented in the patterns of the caloric responses by the algebraic summations shown on the right of fig. 4. In bilateral disorders crossed combinations also occur, and these are shown at the bottom of fig. 4.

The application of the test results to the diagnosis of vestibular lesions is based primarily upon a number of statistical studies of the caloric responses in normal subjects. In figs. 5 and 6 are shown the results obtained by Hallpike, Harrison & Slater (1951) in a group of 93 normal subjects in respect of canal paresis and directional preponderance. In both, the mean value is very close to zero, with standard deviations which have the reasonably low value of 15 seconds. Confirmation of these findings has been provided by the comparable studies of Petermann, Thomsen and others.

FIG. 5. CALORIC TEST RESULTS. FREQUENCY DISTRIBUTION CURVE FOR 93 NORMAL SUBJECTS IN RESPECT OF CANAL PARESIS



Hallpike, Harrison & Slater (1951) by permission of Acta oto-laryngologica, Stockholm

Difference in sensitivity of left and right external canals: represented by responses (1 + 3) − (2 + 4)

Abscissae: duration (sec.)

Ordinates: number of cases

Statistical data: mean value: −1.93
standard deviation: 15.5
probability: 0.2

Interpreted upon this basis, the caloric test results are of high significance in the diagnosis of organic affections occurring at all levels of the vestibular system. Thus, abnormal responses are encountered in some 90% of cases of Ménière's disease of the labyrinth (endolymphatic hydrops), while in tumours of the eighth nerve and in vestibular neuronitis, an important and common toxic affection of the vestibular neurones, the incidence rate of abnormal responses comes close to 100%. In all of these conditions the character of the abnormalities, though varied, is maintained within certain limits. Thus, one or other of the primary deviations from the normal pattern is usually found alone. Alternatively they may occur in combination. Directional preponderance of the responses is also a valuable diagnostic sign of lesions of the vestibular areas within the cerebral hemispheres or brain-stem. Thus, de Barenne & de Kleyn in 1923 first drew attention to the fact that a large unilateral cerebral lesion might bring about such a directional preponderance to the side of the lesion, and the later studies of Fitzgerald & Hallpike (1942), and of Carmichael *et al.* (1954), have since demonstrated that the area of the cortex responsible for this disturbance lies in the posterior part of the temporal lobe.

In addition, Carmichael, Dix and Hallpike have recently carried out a detailed study (unpublished) of the abnormalities of the caloric responses encountered in unilateral vascular lesions of the vestibular centres within the brain-stem. In the majority of their cases directional preponderance to the opposite side was found to occur in association with lesions of the vestibular nuclei lying below the point of entry into the brain-stem of the vestibular nerve. Canal paresis was less common, being encountered only with lesions at or above this level. These findings provide interesting data for speculation upon the anatomical substratum within the brain-stem, of the phenomenon of directional preponderance.

In summary, it may be said that the caloric tests are much the most useful of the clinical tests of vestibular function, provided that the test procedure specified is followed and the results interpreted in accordance with the principles prescribed.

Two features of the test upon which its success is particularly dependent are:

i. the stimulus is applied in a controlled manner to single known end-organs;
ii. the responses obtained in a particular subject are remarkably stable, and are not altered on repetition by habituation, as may occur, as described by Hood & Pfaltz (1954), in the case of the rotational tests.

The tests are easy to apply and cause little discomfort. Quantitatively, they are very delicate; that is to say, they make

possible the detection and differentiation of small degrees of sensitivity loss. Qualitatively, too, they are very penetrating, and the various qualitative abnormalities which they reveal can be effectively applied to the diagnosis of organic lesions at many levels of the vestibular system, from the labyrinth to the temporal lobe.

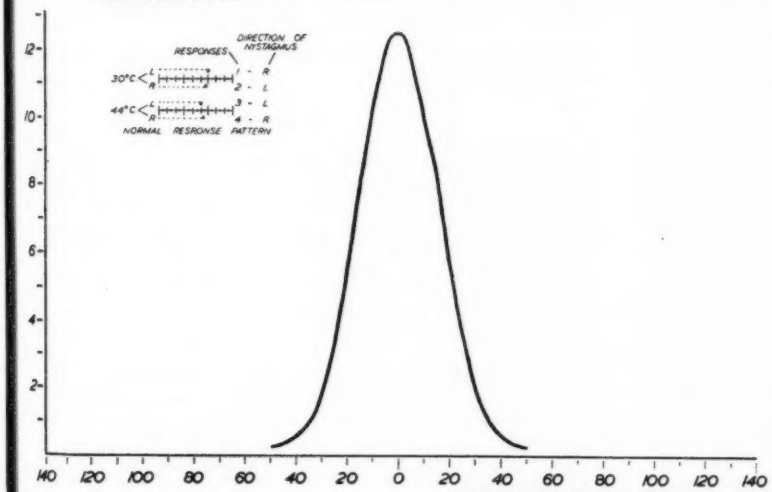
Reference must also be made to difficulties of execution and interpretation, which are well known and need to be borne in mind. Thus, the measurements call for a visual estimation of what is commonly known as the end-point of nystagmus, and the results obtained will depend on the conditions of observation, e.g. adequate illumination. Better standardization of the results is obtainable if it is appreciated that no true end-point is observed, but, rather, the time-point at which the nystagmus ceases to be visible to the unaided eye at a distance of 10 in.[1]

Other difficulties, more serious though in part theoretical, attach to the acceptance of duration as a measure of the magnitude of the nystagmic responses in preference to such other attributes as frequency and amplitude. It is clear, for instance, that a serious quandary would arise if quantitative comparisons were needed between short nystagmic reactions of large amplitude and long reactions of small amplitude.

In practice, this quandary very seldom arises, since, in any given individual, the larger of the responses are also, with few exceptions, the longer; and since the diagnostic value of the tests is dependent upon an analysis of the over-all pattern of the four responses, it follows that their duration would appear to provide for this purpose a reliable measure of their magnitude.

[1] 1 in. = 2.54 cm.—ED.

FIG. 6. CALORIC TEST RESULTS. FREQUENCY DISTRIBUTION CURVE FOR 93 NORMAL SUBJECTS IN RESPECT OF DIRECTIONAL PREPONDERANCE



Hallpike, Harrison & Slater (1951) by permission of Acta oto-laryngologica, Stockholm

Directional preponderance represented by responses (1 + 4) − (2 + 3)

Abscissae: duration (sec.)

Ordinates: number of cases

Statistical data: mean value : −0.15
standard deviation: 15.7
probability: 0.01

The practical advantages of the caloric test procedure, carried out and interpreted in the manner described, do not preclude the need for further development, for which we must await improvements in the technique of nystagmus recording which should make possible a full and rapid analysis of the entire course of the eye movements that constitute a nystagmic reaction.

For the electrical recording of nystagmus, electro-nystagmography, as it is now commonly practised, use is made of the corneo-retinal potentials in accordance with the technique originally described by Miles (1939). The electrical equipment needed is complex and needs to be of high quality to give accurate records of the eye movements, particularly at amplitudes near to the threshold of direct visibility. Recent developments have been reported by Mittermaier (1954), by Montandon (1954) and by Henriksson (1955), to whose publications reference may be made.

The optical recording of nystagmus, by means of the corneal reflex, is also a method of considerable value. Visual fixation is preserved, the eye movements are unrestricted and no electrical equipment is required. The equipment, if suitably designed, is robust and practical. Its sensitivity, too, is considerable and, as Kristensen & Zilstorff-Pederson (1953) have shown, records of caloric nystagmus obtained by this means make possible an estimate of its duration at least as accurate as that obtainable by direct observation.

C. ROTATIONAL TESTS

As explained in the preceding section B, the caloric tests derive their clinical value from the fact that standardized stimuli are applied in ordered succession to particular end-organs of particular labyrinths. The responses thereto reveal small differences of sensitivity or directional tendencies which are found in practice to possess high diagnostic significance. But the physical forces engendered by the stimuli within the canals cannot be readily computed, and hence the method has yielded comparatively little information upon the exact physical characteristics of the canal mechanism.

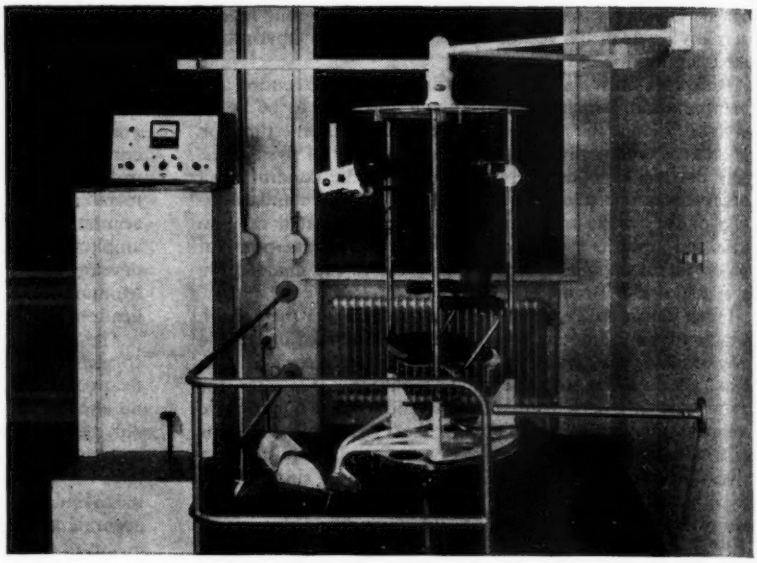
With the rotational tests, however, a different situation is encountered. Both labyrinths are stimulated simultaneously. Hence, deviations from the normal response are difficult to interpret in terms of disordered function of a particular labyrinth —still less of a particular sense organ—and to this extent, therefore, the diagnostic value of the rotational tests is still limited.

In contrast, however, to caloric stimuli, the physical forces engendered within the canals by rotational stimuli can be calculated with considerable precision, a fact which is chiefly responsible for the notable advances made in the last decade in our knowledge of the physical characteristics of the canal mechanism in the normal human subject, advances in which an outstanding part has been played by van Egmond, Groen and their collaborators of Utrecht. The progress so achieved has been intimately dependent upon the development

of high-precision rotating chairs, by means of which it is possible to apply angular accelerations of known duration and magnitude.

In fig. 7 is shown a detailed reproduction by LKB-Produkter, Stockholm, of a chair of this type developed at the Otological Research Unit of the Medical Research Council (Byford, Hallpike & Hood, 1952). By its means, a wide range of angular accelerations and durations is available, 0.1°sec.$^{-2}$ for 1,500 seconds to 10°sec.$^{-2}$ for 15 seconds, with intermediate values between these limits. In addition, provision is made for abrupt stopping from or starting to any desired constant velocity which can, thereafter, be maintained indefinitely.

FIG. 7. REPRODUCTION BY LKB-PRODUKTER, STOCKHOLM, OF ROTATING CHAIR DEVELOPED AT THE OTOLOGICAL RESEARCH UNIT OF THE MEDICAL RESEARCH COUNCIL



An optical system provides a stationary illuminated and magnified image of the eye for the observation of vestibular nystagmus during rotation and, in addition, contacts are provided for electroencephalography and the electrical recording of ocular nystagmus, and other physiological activities.

It is the aim of this communication to review briefly the recent advances that have been made in our knowledge of semicircular canal function by the use of chairs of this type, and in so doing to emphasize certain phenomena peculiar to rotational responses which call for special attention both in the design of test procedures and in the interpretation of the results.

Until the work of Steinhausen in 1931, the long after-sensation of turning which follows the cessation of angular acceleration of the head presented a problem which was resolved by his direct observations upon the cupula of the pike. By means of these he was able to demonstrate that during angular acceleration the inertia of the endolymph caused the cupula to be deflected. When the stimulus was removed, i.e., when the acceleration was followed by a period of rotation at constant velocity, the deflected cupula returned slowly to its rest position under the action of its own restoring force, and it is to this slow return that the long after-sensations of turning could be attributed.

Upon these observations Steinhausen based his well-known torsion pendulum concept of the cupular mechanism, a concept which has exerted a profound effect upon the further development of our views upon the subject. van Egmond and his colleagues advanced this concept by formulating a precise mathematical treatment of the cupulo-endolymph system, supported by a well-known series of experimental investigations in the human subject (van Egmond, Groen & Jongkees, 1949) and in the ray (Groen, Lowenstein & Vendrik, 1952).

They took as the basis of their mathematical treatment the differential equation of the torsion pendulum where there are no external forces:

$$\ddot{\xi} + \frac{\pi}{\theta}\dot{\xi} + \frac{\Delta}{\theta}\xi = 0 \qquad (1)$$

where θ = moment of inertia of the endolymph

π = moment of friction for unit angular velocity

Δ = restoring force for unit angular deviation of the cupula

ξ = the angular deviation of the endolymph in relation to the skull

$\dot{\xi}$ = the angular velocity of the endolymph in relation to the skull

$\ddot{\xi}$ = the angular acceleration of the endolymph in relation to the skull.

van Egmond and his collaborators established the values of the constants as follows:

$$\frac{\pi}{\Delta} = 10; \frac{\theta}{\Delta} = 1.$$

The significance of their contribution arises from the fact that the behaviour of the cupula in response to any given rotational stimulus could be theoretically predicted. For example, the approximate solution of the above equation relating to impulsive stimulation, that is to say, a sudden stopping of the chair from a constant velocity γ is

$$\xi = \gamma\frac{\theta}{\pi}\left[e^{-\frac{\Delta t}{\pi}} - e^{-\frac{\pi t}{\theta}}\right] \qquad (2)$$

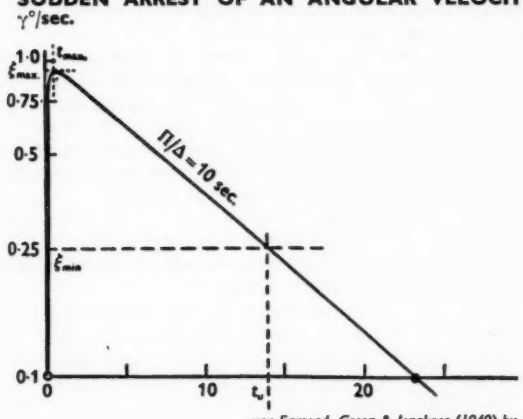
with the limiting conditions: $t = 0$, $\xi = 0$ and $\dot{\xi} = \gamma$.

The behaviour of the cupula under these conditions is shown graphically in fig. 8. The slow return of the deflected cupula is determined by the first term in equation (2) $e^{-\frac{\Delta t}{\pi}}$ so that, if one assumes that the after-sensation expires when the cupula has reached a minimal perceptible deflection $\xi_{min.}$, then the duration of the after-sensation t_u is given by

$$t_u = \frac{\pi}{\Delta}\log\left(\frac{\theta\gamma}{\pi\xi_{min.}}\right) \text{(approx.)} \qquad (3)$$

It follows from this equation that, if a number of determinations are made of the after-sensation durations using

FIG. 8. DEVIATION ξ OF CUPULA PRODUCED BY SUDDEN ARREST OF AN ANGULAR VELOCITY



van Egmond, Groen & Jongkees (1949) by permission of Journal of Physiology

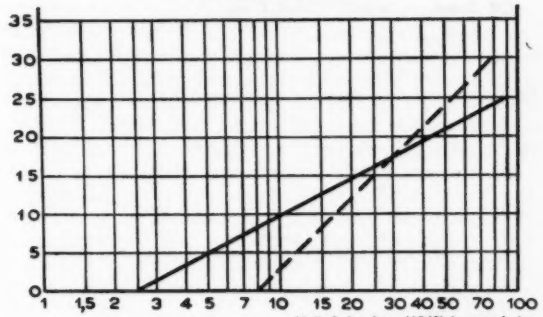
Maximum deviation occurs at 0.5 sec.
Minimum perceptible deviation occurs at 14 sec.
Abscissae: time (sec.)
Ordinates: cupular deviation (logarithmic scale)
Slope of curve gives $\frac{\pi}{\Delta}$

different stopping velocities, then a logarithmic plot of the impulse against time should yield a straight line curve, the slope of which is the constant $\frac{\pi}{\Delta}$.

van Egmond *et al.* (1949) refer to this graph as a cupulogram, and to the tests required for its determination as cupulometry. They have used it extensively not only for the determination of the constant $\frac{\pi}{\Delta}$ in normal subjects, but also in the clinical investigation of diseases of the vestibular system, where deviations from the normal might be expected to provide results of diagnostic significance. The after-nystagmus is measured and plotted in the same way, and the two kinds of cupulogram so obtained are shown in fig. 9, reproduced from a paper by Hulk & Jongkees (1948). The nystagmus cupulogram does not have the same slope as the sensation cupulogram, and for this reason they suggest that the two curves are independent expressions of the activity of the vestibular organ.

A practical demonstration of the torsion pendulum concept of the canal mechanism, with confirmation of its physical constants as determined by van Egmond and his collaborators, is readily achieved by means of the equipment shown in fig. 7. Thus, the response of the cupula, calculated upon the basis of Groen's equation to a sustained acceleration of 5°sec.$^{-2}$ is shown by the curve of fig. 10. If, after 5 seconds, when the deflection has reached 2°, the acceleration is replaced by a period of constant velocity, the cupula will be returned, in the manner shown, to its resting position by its elastic restoring force, in a period corresponding to that of the after-sensation. It is clear that for every value of terminal deflection along this curve there must be a corresponding value for the restoration time, i.e., the after-sensation duration. Conversely, from a knowledge of the latter it is possible to calculate the value of the former, and this can be done upon the basis of the following simple series of observations.

FIG. 9. AFTER-SENSATION AND AFTER-NYSTAGMUS CUPULOGRAMS



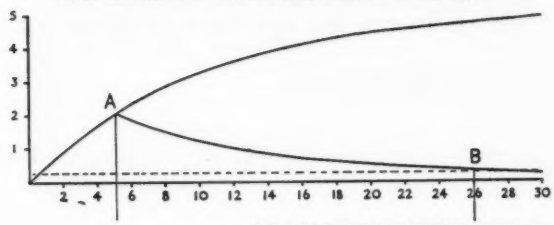
Hulk & Jongkees (1948) by permission of Journal of Laryngology

Abscissae: impulse (deg. sec.)
Ordinates: duration (min.)
——— average normal after-sensation cupulogram
– – – – – average normal after-nystagmus cupulogram

Accelerations of 3°sec.$^{-2}$, 5°sec.$^{-2}$ and 7°sec.$^{-2}$ are applied for periods of 2 seconds, 4 seconds, 6 seconds and 8 seconds, and the after-sensation durations measured at each test. From these the terminal cupular deflections are calculated so that in this way it is possible to establish the magnitude of the deflection reached by the cupula during sustained acceleration at each of the periods given above. The results of this procedure are shown in fig. 11, together with the theoretical deflection curves, and it will be seen that there is good coincidence between them. By this means, therefore, it is possible to confirm the validity of the torsion pendulum concept and to verify the values assigned to its constants by van Egmond and his collaborators.

In the course of their work, van Egmond and his co-workers made extensive use of impulsive stimuli. They accepted the after-sensation duration as a measure of the cupular restoration time, and took their results to represent the mechanical characteristics of the cupulo-endolymph system. Here certain artificialities are apparent since, in the human subject, such results must clearly be the resultant of the constants, not only of the cupulo-endolymph system itself, but also of the entire vestibular nervous complex which connects it with the cerebral hemispheres. Thus, the torsion pendulum concept

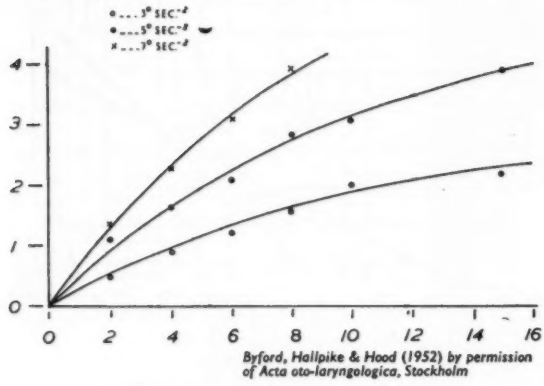
FIG. 10. CUPULAR DEFLECTION OCCURRING IN RESPONSE TO ACCELERATION OF 5°sec.$^{-2}$



Byford, Hallpike & Hood (1952) by permission of Acta oto-laryngologica, Stockholm

Abscissae: time (sec.)
Ordinates: cupular deflection (deg.)
A – B: after-sensation

FIG. 11. THEORETICAL CUPULAR DEFLECTIONS OCCURRING IN RESPONSE TO ACCELERATIONS OF 3°sec.$^{-2}$, 5°sec.$^{-2}$ and 7°sec.$^{-2}$



Byford, Hallpike & Hood (1952) by permission of Acta oto-laryngologica, Stockholm

Abscissae: time (sec.)
Ordinates: cupular deflection (deg.)

cannot be validly applied to the system as a whole without some assumption of a mutual reciprocity of the constants of its various elements, both neural and mechanical. Although this assumption would, in many ways, appear justified, an obvious need exists to envisage the occurrence of disturbances of the over-all responses resulting from purely central influence, disturbances which could not validly be interpreted in terms of physical changes in the cupulo-endolymph system.

An example of this is provided by the well-known habituation phenomenon, whereby the subjective and reflex responses to rotation may undergo severe reduction as the result of simple repetition, a phenomenon dependent almost certainly upon central processes without alteration of the physical behaviour of the peripheral mechanism.

In previous investigations of this phenomenon in man and animals, the rotational stimuli used have been excessive, and there is good reason to believe (Groen & Jongkees, 1948) that pathological derangement of vestibular function must have played an important role in the results obtained. In a recent series of experiments upon rabbits, however, Hood & Pfaltz (1954) used stimuli well within the physiological range. Their experimental test procedure will be seen from fig. 12. With the animal's head lowered some 30° below the horizontal, in order to bring its horizontal canals into the plane of maximal reactivity, it was subjected to a standard test consisting of an angular acceleration of 5°sec.$^{-2}$ for 10 seconds, followed by a period of rotation at constant velocity for 60 seconds, whereupon the chair was decelerated to rest at 5°sec.$^{-2}$. The animal's eye was kept under continued observation during this time, by means of the optical system shown in fig. 7, and a record was made of the nystagmic beats during and following the acceleration and the deceleration. The tests were applied alternately with rotation to the right and the left, and a summary of the results obtained in three typical animals is shown in fig. 13. These were tested as follows:

Rabbit No. 1. One standard test, giving two stimuli (acceleration and deceleration), was applied repeatedly at intervals of 1 minute for three periods of 30–45 minutes during a total period of 24 hours.

Rabbit No. 2. A succession of four standard tests at intervals of 1 minute was applied daily for seven days.

Rabbit No. 3. A succession of four standard tests at intervals of 1 minute was applied weekly for seven weeks.

The average values, expressed in terms of the number of nystagmic beats, of the results of every four successive stimuli are shown plotted in numerical order upon the abscissae. It will be seen that, in all three animals, repeated tests resulted in a considerable reduction in the responses. Though the time scales of the three experiments were very different, the curves obtained were very similar, and this can be taken to indicate that the physiological mechanism, upon which is dependent the fall of the responses, is in some way capable of summing the effects of all preceding stimuli, irrespective of the time interval between them.

The phenomenon has, in the past, come to be known as habituation. Though little is known of its physiological mechanism it would appear almost certain to be central in origin. Its practical importance as a source of error in the quantitative assessment of the results of rotational tests in human subjects requires no emphasis.

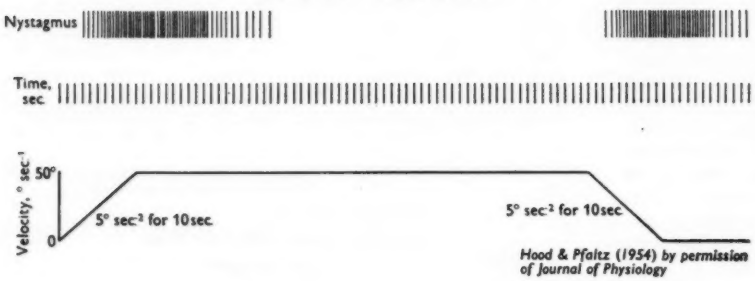
By contrast the caloric responses in rabbits resist modification by repetition, a finding in good agreement with experience of repeated caloric tests in human subjects.

The Oculo-Gyral Illusion

In all of the test procedures so far described the duration of the after-sensation of turning has been accepted as a measure of the return time of the cupula to its rest position. We may now consider an alternative technique based upon the oculo-gyral illusion which enables direct measurements to be made of the magnitude of the sensation of rotation at all times during and following the application of a rotational stimulus. The method has been used to arrive at an independent evaluation of the constants of the cupulo-endolymph system. It has one notable advantage, namely, that by its means it is possible to avoid the distorting effect of habituation.

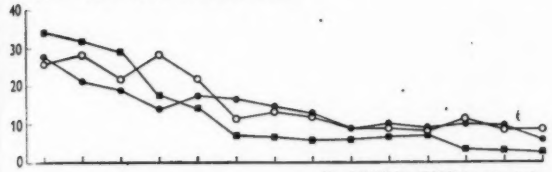
The illusion is described as follows: a subject seated in darkness is required to fixate a small light spot fixed to the chair at a convenient distance in front of him. Under these conditions any acceleration of the chair to the left or right provokes a sensation of movement of the light, which corresponds in respect of direction, velocity and duration to the sensation of turning itself.

FIG. 12. NYSTAGMIC RESPONSE OF RABBIT TO ACCELERATION OF 5°sec.$^{-2}$ FOR 10 sec.



Hood & Pfaltz (1954) by permission of Journal of Physiology

FIG. 13. NYSTAGMIC RESPONSES OF THREE RABBITS TO SUCCESSIVE ACCELERATING STIMULI APPLIED AT VARYING INTERVALS



Hood & Pfaltz (1954) by permission of Journal of Physiology

Abscissae: successive groups of 4 responses
Ordinates: number of nystagmic beats per response, expressed as average for successive groups of 4 responses

According to Graybiel, Clark, McCorquodale & Hupp (1946), the illusion depends upon a tracking of the image of the light source across the retina during the slow component of the vestibular eye nystagmus induced by cupular deflection. According to Lorente de Nó (1931) and others the speed of the slow component is a function, and provides a measure, of the cupular deflection. It follows that the complete course of the cupular response to a rotational stimulus could be provided by a continuous record of the slow component velocity, and Hallpike & Hood (1953) have made use of the oculo-gyral illusion to obtain such records.

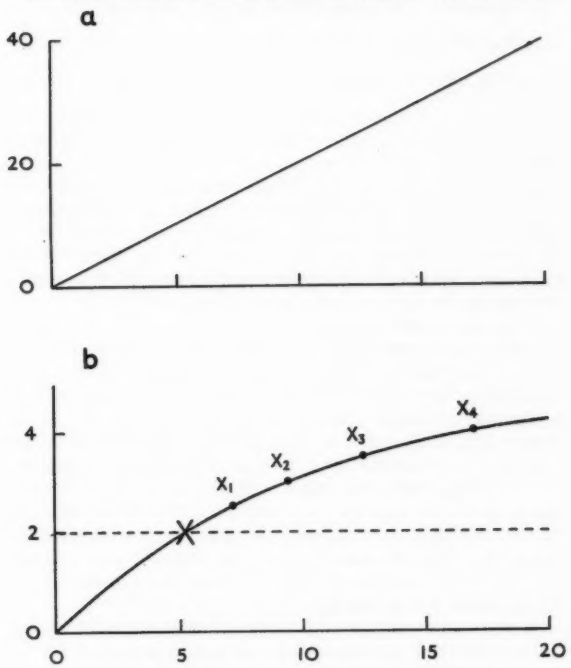
The rationale of their test procedure is illustrated schematically in fig. 14. With constant acceleration to the right (fig. 14a), the light spot will appear to move to the right with a velocity which increases with time. A rough subjective estimate of the time-course of this velocity is shown in fig. 14b. An exact evaluation of this curve is achieved by means of the following simple cancellation technique. At the beginning of the test the light spot is made to move with constant velocity of, say, 2°sec.$^{-1}$ to the left, and at this moment its actual movement will be opposed to that of the subsequent illusory movement. With increasing deflection of the cupula and corresponding increase of the speed of the slow nystagmus component a point will be reached at which the latter will equal the actual angular velocity of the light itself with cancellation of the sensation of its movement. This point is shown at X on fig. 14b. With a higher actual velocity of light movement this cancellation point will occur later, and can be similarly determined. Thus, with actual velocities of 2.5°sec.$^{-1}$, 3°sec.$^{-1}$, 3.5°sec.$^{-1}$ and 4°sec.$^{-1}$ the cancellation points are found to occur at X_1, X_2, X_3, X_4, and the curve so obtained can accordingly be taken to represent the course of the cupular deflection.

Hallpike and Hood were able in this way to establish values for the constants of the cupulo-endolymphatic system which were in close agreement with those given by van Egmond and his collaborators. As with cupulometry, however, the method was found to be attended by certain disadvantages. Thus, the number of tests required to determine the complete cupular response to a particular stimulus was time consuming. In addition, the possibility of error due to habituation was not excluded. These defects have been eliminated by means of a technique (Hood, unpublished) which provides a continuous record of the response to a single stimulus. In this procedure the light stimulus consists of a number of vertical illuminated lines which are made to move to the right or left at a velocity which is continuously recorded.

The following is the procedure employed in recording the subjective response to an impulsive stimulus. Beginning with the chair at rest, the light is caused to move to the left with a small fluctuating velocity so adjusted that the peaks upon the record obtained give a measure of the threshold of perceptible movement. The impulsive stimulus is now applied and the chair made to attain a constant velocity to the right of 40°sec.$^{-1}$ within 0.5 second. At this point the directions of the real and illusory motion of the light are opposed. The velocity of the latter exceeds that of the former which in consequence needs to be increased before the subject again reports minimal movement to the left. By means of this cancellation procedure an instantaneous measure of the velocity of the apparent movement of the light is obtainable at this point. The sensation of rotation diminishes with time, its fall being matched by a fall in the velocity of the apparent movement of the light, the full course of which can be continuously measured and recorded by means of the same cancellation technique.

The typical record shown in fig. 15 may be considered in relationship to the theoretical course of the cupular deflection. The response of the cupula to a stimulus of this kind has been shown in fig. 8. We are concerned here only with the decline

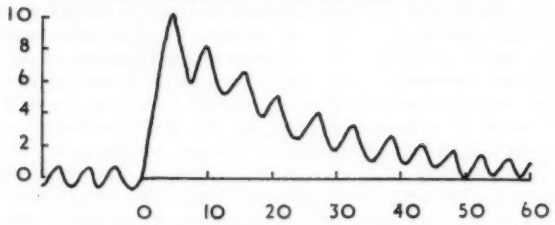
FIG. 14. RATIONALE OF TEST PROCEDURE BASED ON OCULO-GYRAL ILLUSION (Hallpike & Hood, 1953)



a: time course of angular velocity of chair to right (2° sec.$^{-1}$)
Abscissae: time (sec.)
Ordinates: chair velocity (deg. sec.$^{-1}$)

b: curve showing cancellation of actual motion of light to left (interrupted line) by illusory motion of light to right (continuous line) induced by constant acceleration to right
Abscissae: time (sec.)
Ordinates: light velocity (deg. sec.$^{-1}$)

FIG. 15. TYPICAL RECORD OF SUBJECTIVE RESPONSE TO AN IMPULSIVE STIMULUS OF 40°sec.$^{-1}$



Abscissae: time (sec.)
Ordinates: light velocity (deg. sec.$^{-1}$)

expressed by equation (2). The only two variables in this equation are t and ξ and it is clear that where ξ is plotted logarithmically against t we obtain a straight line, the slope of which gives the constant $\frac{\pi}{\Delta}$. Now, if the magnitude of the oculo-gyral illusion, as measured by the apparent velocity of the light, is directly proportional to the deflexion,[2] then the values for this light velocity, experimentally determined in the manner described and plotted logarithmically against time, should yield a straight line curve, the slope of which is again the constant $\frac{\pi}{\Delta}$. The result is shown in curve 1, fig. 16, which is derived from the record shown in fig. 15. It will be seen that a straight line curve is obtained with a slope $\frac{\pi}{\Delta} = 24$.

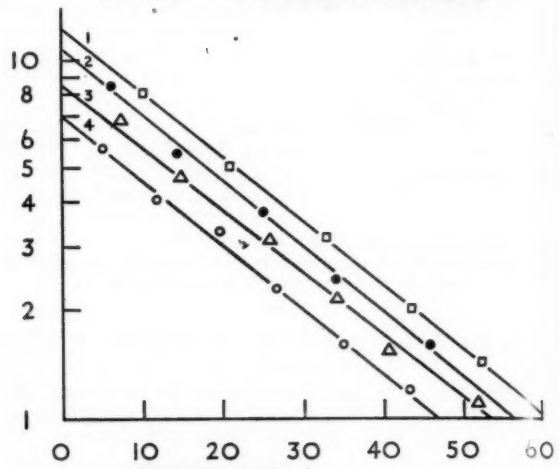
This new technique thus makes possible the evaluation of the constants of the semicircular canal mechanism from its response to a single stimulus. The importance of this is considerable; thus, on theoretical grounds the evaluation of the constants from the responses to a series of stimuli, as with the conventional cupulometric procedure, is invalidated by the fact that each point on the curve would be determined in part by the habituation effect of preceding stimuli to an extent determined by their number and intensity.

This is exemplified in fig. 17. The solid curve A–B is taken to be the true cupulogram, i.e., that obtained without distortion by habituation. Since, in fact, a number of stimuli of increasing intensity are applied, the response to each must be successively reduced, and the curve obtained will thus be the dotted line A–C, with a corresponding departure from the true value of the constant. This distorting effect of habituation on cupulometry has been emphasized by de Wit (1953), and indeed it would not seem too much to say that this important source of error is likely to apply to any test procedure involving repeated stimuli. It follows that the correct value of the constant can be derived only from the full analysis of the response to a single stimulus.

A true picture of the cupular response to repeated stimuli is given by the complete set of four curves shown in fig. 16. Four successive stimuli have been applied and from each response an independent evaluation of the constant has been obtained. It will be seen that, although the response

[2] It is necessary to stress that the exact mechanism of the illusion has not yet been fully determined. Hallpike & Hood (1953) have pointed out that the illusion may be present in the absence of visible nystagmus. van Dishoeck, Spoor & Nijhoff (1954), too, have adduced evidence which would appear to controvert the retinal tracing theory of Graybiel *et al.* (1946). In spite of these difficulties it would seem likely that the illusion has as its physiological basis the deflection of the cupula. Hence, the technique described provides, in all probability, an acceptable method for the quantitative investigation of cupular function.

FIG. 16. RESPONSES TO FOUR IMPULSIVE STIMULI OF 40°sec.$^{-1}$ APPLIED IN SUCCESSION



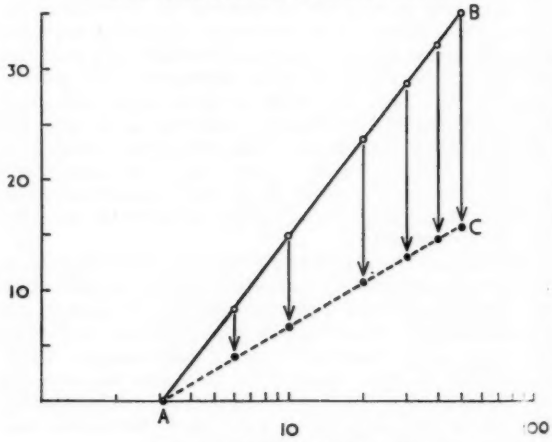
Abscissae: time (sec.)
Ordinates: light velocity (deg. sec.$^{-1}$)

falls with repetition of the stimulus, nevertheless the slope of the curve and hence the value of the constant is entirely unchanged.

D. POSITIONAL TESTS OF OTOLITH FUNCTION

The otolith organs, regarded as physical instruments, come within the category of linear accelerometers. The acceleration to which they are normally subject is that due to gravity. Their responses vary with their inclination and thus they signal the orientation of the head.

FIG. 17. DISTORTING EFFECT OF HABITUATION UPON SLOPE OF CUPULOGRAM



Abscissae: impulse (deg. sec.$^{-1}$)
Ordinates: duration (sec.)

AB: probable true form of cupulogram, without distortion by habituation

AC: cupulogram derived from responses to successive stimuli of increasing intensity

Clinical tests of otolith function are notoriously unsatisfactory. For this the chief reason is perhaps the fact that stimuli cannot be limited to any particular otolith organ, or indeed to the organs of one side. Hence, functional defects can be demonstrated by means of the tipping table or horizontal swing only when severe bilateral damage has occurred.

In 1921, Bárány drew attention to certain remarkable clinical phenomena which appeared to be due to disordered function of the human otolith apparatus. He described the case of a patient in whom severe vertigo and nystagmus was brought about by a particular position of the head, namely, supine with the head to one side. The nystagmus was brisk and of a particular kind, rotatory, with a horizontal element towards the undermost ear. Bárány demonstrated that the factor which precipitated the phenomena was not movement but position of the head in space, and attributed them for this reason to a disorder of the otolith apparatus.

Bárány also noted that the nystagmus and vertigo were subject to adaptation, and disappeared rapidly if the critical head position was maintained. No other evidence of ear disease was present in Bárány's patient; hearing and the caloric responses were normal, and neurological examination revealed nothing abnormal.

This clearly described clinical picture of paroxysmal vertigo and nystagmus related to a particular head position, occurring in an otherwise healthy patient, has been the subject of numerous clinical studies, and the findings of Gerlings (1948) and some others have strongly suggested, in general agreement with Bárány's views, that its pathological basis is a lesion of the internal ear, an opinion which has now received substantial confirmation from the recent clinico-pathological studies of Dix & Hallpike (1952) and of Cawthorne & Hallpike (unpublished).

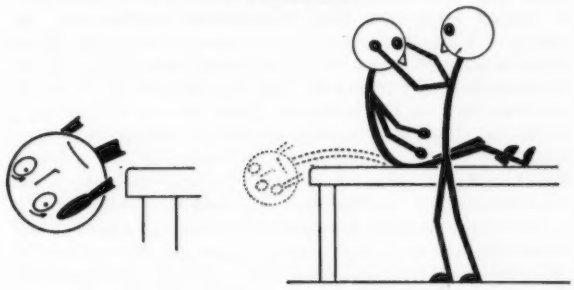
Dix and Hallpike, in order to emphasize the paroxysmal character of the nystagmus and the benign course of the disorder, have termed it the benign paroxysmal type of positional nystagmus. The character of the nystagmus and the procedure used to elicit it are depicted in fig. 18. The critical position is defined as follows: The patient is laid supine with the head lowered some 30° below the horizontal, and turned some 30°–45° to one side. In taking up this position the patient is first seated upon a couch with the head turned to one side. The examiner then grasps the patient's head and takes it back briskly into the critical position. A latent period of a few seconds usually precedes the onset of the nystagmus, which is accompanied by vertigo and feelings of general distress, as Bárány stated. It generally adapts rapidly and is seen only in a diminished form if the test is repeated without a long interval.

This, the benign paroxysmal variety of positional nystagmus, is not the only one observed by Bárány. He made mention also of another, in which the nystagmus showed less adaptation with maintenance of the critical head position.

In addition to the absence of adaptation, this variety of positional nystagmus is often characterized by the fact that its direction changes with alterations of head position. For this reason Nylén (1931) termed it the direction-changing variety of positional nystagmus. Nylén was able to show that it is a common accompaniment of deep lesions of the central nervous system within the posterior fossa, and of such indeed he justly claimed that it provided valuable diagnostic evidence.

Whether the otolith system is affected at its periphery, or within its central connexions, with appropriate variations in the character of the positional nystagmus, the occurrence of this nystagmus may be the only physical sign of organic disease, not only of the vestibular, but indeed of the whole nervous system. This fact and the simplicity of the test procedure make it one of the most important of all the clinical tests of vestibular function, a point of view long and cogently sustained by Nylén, the pupil and successor of Bárány in the Chair of Otology at Uppsala.

FIG. 18. PROCEDURE USED TO ELICIT POSITIONAL NYSTAGMUS



E. TESTS FOR OPTOKINETIC NYSTAGMUS

Two good reasons are offered for including tests of optokinetic or railway nystagmus in the diagnostic armamentarium of the neuro-otologist: firstly, the introduction by Bárány himself of his well-known striped rotating drum; secondly, the fact that certain of its characteristics have long claimed for it an intimate relationship to the vestibular system. Thus, it exhibits the same alternation of slow and rapid components so characteristic of vestibular nystagmus.

Now according to Dohlman (1938) and others, the rhythm and waveform of vestibular nystagmus is dependent upon the neurological properties of the vestibular nuclei, and Ohm (1936) has accordingly argued with some persistence that the latter constitute a cell station common to the reflex pathways of both varieties of nystagmus. This view has now been rendered untenable by a substantial body of clinico-pathological evidence. Thus, the animal experimental work of Winston and his colleagues (1948) has made it clear that the vestibulo-toxic effects of streptomycin are dependent, at any rate in part, upon destruction of the central vestibular neurones (Winston, Lewey, Parenteau, Marden & Cramer, 1948).

Subsequently, Dix, Hallpike & Harrison (1949) were able to show in a series of human subjects with severe degrees of streptomycin intoxication that, although the caloric responses were absent or very severely reduced, the optokinetic responses were, without exception, brisk and normal. This proof that the reflex pathways for optokinetic nystagmus are independent of the vestibular nuclei has since received support from the results of a more detailed study by Blackwood, Carmichael & Hallpike (unpublished) of the temporal bones, eighth nerves and brain-stem of a case of advanced sensory neuronopathy. In this material, extensive degenerative changes were found to be present, both in the peripheral vestibular neurones and also in the vestibular nuclei within the brain-stem. Here again, though the caloric responses were absent, the optokinetic responses were normal.

Clinical evidence concerning the cortical control of vestibular and optokinetic nystagmus has also made it clear that at higher levels too their neurological mechanisms are distinct. Thus, the cortical areas concerned with the directional control of caloric nystagmus have been shown by the investigations of Carmichael, Dix and Hallpike to lie in the posterior part of the temporal lobes, whence they exert a regulating effect upon caloric nystagmus occurring in particular directions. This they are thought to do by virtue of their modifying influence upon the reflex reactivity of the vestibular nuclei. The findings of Carmichael, Dix & Hallpike (1954) have also confirmed the earlier views of Stenvers and some others that certain cortical areas are also concerned with the same sort of directional control of optokinetic nystagmus. These areas have been shown to lie in the supramarginal and angular gyri of each hemisphere; and each is thought to exert a controlling action upon optokinetic nystagmus occurring in a particular direction; again, by virtue of a modifying influence upon lower reflex centres, located, in all probability, in the superior colliculi.

Although these investigations have deprived optokinetic nystagmus of its supposed vestibular affinities, they have also served to clarify and enhance its clinical significance, and hence to maintain its diagnostic value to the neuro-otologist. Observations of the responses evoked by means of the Bárány drum are made by direct inspection, and their purpose is to estimate the amplitude and regularity of the eye movements and, in particular, their symmetry in the vertical and horizontal planes.

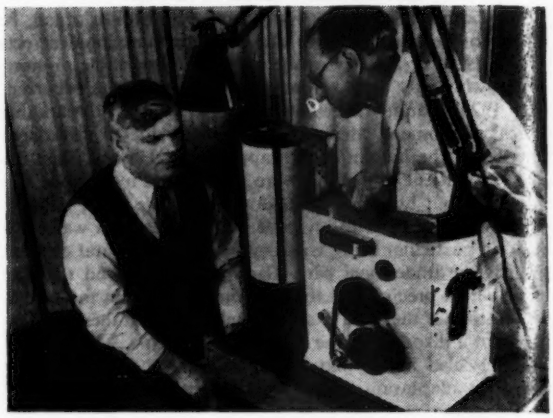
Satisfactory results call for care in the design and construction of the equipment in respect of the following requirements:

i. accurate speed control with rapid reversibility of the drum;

ii. ease of observation of the nystagmus.

A satisfactory type of equipment is shown in fig. 19. Three drum speeds are provided: 360°/sec., 180°/sec., and 72°/sec.

FIG. 19. BÁRÁNY DRUM USED TO TEST OPTOKINETIC NYSTAGMUS



The angle of the drum axis is continuously variable, and it is thus possible to investigate nystagmus in any desired direction. Both the speed control and the reversal switch can be operated instantaneously by the examiner without relaxing his scrutiny of the subject's eyes, which are well illuminated. In this way it is possible to make very fine and rapid judgements of the symmetry of the eye movements in different planes.

Tests of optokinetic nystagmus are of particular value in the diagnosis of cerebral lesions. Thus, a unilateral lesion involving the supramarginal and angular gyri is characteristically associated with a weakening or suppression of the nystagmus to the opposite side. In addition, derangements of the responses are common manifestations of organic lesions involving the optokinetic pathways at lower levels within the brain-stem, in particular, intramedullary neoplasms and disseminated sclerosis.

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THE CONTRIBUTION OF SURGERY TO THE PROBLEMS OF NEURO-OTOLOGY

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1 Ménière's disease
2 Effect of unilateral labyrinthectomy
3 Sound conduction within the labyrinth
4 The Tullio effect
5 Organic lesions of the central connexions of the eighth nerve system and their localization by surgery
References

It was by surgical experiments upon the semicircular canals of the pigeon that Flourens (1824) discovered that the internal ear was concerned with balance as well as with hearing. The idea of the labyrinth's serving a dual purpose penetrated slowly, though Ménière in 1861 ventured to attribute a disorder of hearing and balance in man to disease of the inner ear. Since then much information about the end-organs of the eighth nerve in the labyrinth and their connexions within the central nervous system has been collected, mainly as the result of experiments on animals.

In man the increased precision with which operations upon the ear and the central nervous system can now be carried out has added further to our knowledge of cochlear and vestibular function in health and disease, and it will be the purpose of this article to describe some of the ways in which surgery has helped to confirm or modify views put forward as the results of experiments in animals and clinical observations in man.

1. Ménière's Disease

Despite Ménière's discovery, many observers found it hard to believe that fluctuations in the activity of such a modest little organ as the labyrinth could have such a profound and widespread effect upon bodily functions; though Gowers (1893) stated:

> . . . it is exceedingly rare for definite vertigo to occur apart from aural symptoms, and it is certain that, in the majority of cases in which such vertigo has been ascribed to other causes, these have only had an exciting influence, and the symptom has been essentially due to the effect of unobtrusive labyrinthine disease . . .

Lake (1904) described a case of unilateral deafness and repeated attacks of vertigo in which the attacks ceased after removal of the semicircular canals.

However, the frequency with which operations on the middle ear and labyrinth were followed by infection, sometimes with fatal results, did not encourage surgeons to operate on the ear for non-suppurative disorders; though Jenkins (1911) opened the bony lateral semicircular canal for the relief of vertigo, and curiously enough he used the word " fenestration " to describe his procedure.

Later Dandy (1928) advocated section of the eighth nerve within the skull, and he was impressed with the possibility that the attacks might arise in the nerve trunk itself—he used as an analogy the spasmodic bouts of pain in trigeminal neuralgia. The presence of looped blood vessels around the eighth nerve trunk and sometimes between its divisions led him to suggest that pressure on the nerve trunk by blood vessels might also initiate attacks.

Thus there was still some doubt as to the exact locus of the lesion and indeed about its nature, for Ménière's disease pursues a benign course so that material for detailed histological examination was not readily available.

Then in 1938 Cairns & Hallpike were able to study the temporal bones from two subjects of Ménière's disease, in which intracranial section of the eighth nerve had ended fatally. They found distension of the cochlear canal and saccule of the endolymphatic system, and they regarded this endolymphatic distension as the essential feature of Ménière's disease; their findings have since been confirmed by other observers.

This established the site and nature of the disorder. In the meantime Mollison (1931) had reported a series of cases in which injection of alcohol into the labyrinth through an opening in the bony lateral semicircular canal was followed by loss of cochlear and vestibular function and freedom from further attacks.

In 1938 Cawthorne and Hallpike, using a Leitz binocular dissecting microscope (magnification × 10), found that, by simply removing the membranous external semicircular canal in cases of Ménière's disease, all cochlear and vestibular function was destroyed in the labyrinth operated on. A series of cases operated upon in this way was reported in 1943 by Cawthorne. Later it was noted that this procedure's destructive effect upon the labyrinth did not necessarily apply to disorders other than Ménière's disease (Cawthorne, 1949).

Recently Lindsay & Siedentop (1955) have found that nothing short of removal of most of the membranous labyrinth is sufficient to ensure complete loss of function; though, in a series of 288 operations for Ménière's disease, Cawthorne & Hewlett (1954) found that, in all of them, removal of the membranous semicircular canal never failed to achieve total loss of both cochlear and vestibular function.

2. Effect of Unilateral Labyrinthectomy

Once it had been established that labyrinthine function could be abolished without any local complicating factors such as infection or irritation from alcohol, it was possible to observe in man the effect upon eye movements of unilateral destruction of the labyrinth.

First of all, it was found that nystagmus did not appear until the patient had recovered consciousness after the general anaesthetic. Until then no unusual eye movements were noted.

When it appeared, the nystagmus was always with the quick component towards the ear not operated on, and the degree and duration varied in accordance with the amount of preoperative vestibular function; that is to say, when tests before operation revealed only slight impairment, the post-operative nystagmus (and associated symptoms) was severe and might last as long as three weeks. On the other hand, if there was but little response on the affected side to vestibular tests

before operation, the post-operative nystagmus was slight and might last only a few days.

The nystagmus towards the sound side following unilateral labyrinthectomy slowly subsided, until at the end of about three weeks it could no longer be seen. This gradual suppression of the nystagmus had been noted by von Bechterew (1882) who attributed it to the tonic action of the vestibular nuclei in the brain-stem, which allowed the vestibulo-ocular system to adjust itself to its unbalanced state.

By employing the caloric test of Fitzgerald & Hallpike (1942) on the ear not operated on, it was possible to demonstrate that, after unilateral labyrinthectomy, a tendency of nystagmus towards the sound side could be revealed long after the spontaneous nystagmus had subsided. The effect of stimulating the active labyrinth, on the side not operated on, with water equally below and then equally above body-temperature, was to change the normally equal nystagmic response, so that the intensity and duration of the nystagmus following the hot stimulus was prolonged and that after the cold stimulus was shortened. In other words, nystagmus towards the sound side was augmented and that towards the affected side was diminished. This these workers termed " a directional preponderance ", which they regarded as evidence of a lack of balance within the vestibular system as the result of one labyrinth being put out of action.

Now a somewhat similar state of affairs had first been noted by Ewald (1892) in pigeons, after he had made an opening into the lateral semicircular canal of one labyrinth. Applying a pneumatic hammer to the opening, he found that compression caused a flow of endolymph towards the ampulla; this was followed by a much greater response in the form of eye and body movements than the reverse, which caused a flow of endolymph away from the ampulla and a lesser response. Therefore, he concluded that in the lateral canal a flow of endolymph towards the ampulla was a more powerful stimulus than a flow of endolymph away from the ampulla, and this apparent uni-directional sensitivity of the cupula was accepted as a physiological law and became known as Ewald's second law.

Some years later Bárány (1906) found that, in a patient in whom one labyrinth had been previously destroyed by disease, rotation of the patient in the plane of the lateral canal, so that a sudden stop caused a flow of endolymph towards the ampulla of the sound labyrinth, caused a greater after-nystagmus than rotation in the opposite direction with a flow of endolymph away from the ampulla. Thus it appeared to Bárány that Ewald's second law applied to man.

If this were so, then syringing the ear with water above body-temperature, which causes a flow of endolymph towards the ampulla of the lateral canal, should constantly result in a greater nystagmus than syringing with water equally below body-temperature. But Fitzgerald & Hallpike (1942) had shown that in the intact vestibular system in man this never occurs, though, as the result of their observations on the effects of unilateral labyrinthectomy in man, Cawthorne, Fitzgerald & Hallpike (1942) found that caloric stimulation of the normal labyrinth after operation always resulted in a more prolonged nystagmus after the hot stimulus.

This change in the responses on the normal side they regarded as being due to damage to one labyrinth causing a lack of balance between the two vestibular systems.

It could of course have been interpreted as an example of uni-directional sensitivity in the labyrinth tested. But they got such a result only when one labyrinth had been damaged though not necessarily destroyed, and the observations of Ewald and Bárány had each been made on a damaged vestibular system. Therefore they felt that this effect was the result of some abnormality and they stated that Ewald's second law did not apply to the intact human vestibular system.

Using a minimal rotational stimulus " cupulometry ", van Egmond, Groen & Jongkees (1949) also found that Ewald's second law did not apply to the intact human vestibular system, and recently Hallpike & Hood (1953), also using a rotational stimulus, came to the same conclusion.

3. Sound Conduction within the Labyrinth

Otosclerosis has long been a challenge to the aural surgeon. Early attempts to overcome the obstruction to the passage of sound waves into the labyrinth were naturally directed towards the site of the obstruction, but removal of the stapes (Kessel, 1876) was rarely rewarded by success and, indeed, before the end of the nineteenth century this and allied procedures were condemned because of suppuration which sometimes resulted in loss of function and even life.

Then in 1914 Jenkins described three cases of otosclerosis in which he had made an opening into the lateral semicircular canal of the vestibular labyrinth. He thought that this might remove abnormal fluids from within the labyrinth, which might be contributing to the deafness. At the same meeting Bárány described how in 1910 he had opened the posterior semicircular canal and how he felt that, by doing so, the movement of the fluids within the labyrinth in response to sound might be encouraged. Holmgren (1913) opened the superior semicircular canal. However, all these attempts finally failed because the two mobile windows in the bony labyrinth were subjected equally to pressure of the entering sound wave.

Sourdille (1929) finally hit upon the idea of having one window (the new fenestra) open to the incoming sound wave and the other (round window) protected by the tympanic membrane. This open operation was simplified and perfected by Lempert (1938, 1941), and his one-stage procedure is the basis of the modern fenestration operation, which has finally established that in otosclerosis, in which the oval window is impeded, a mobile window on the vestibular side of the basilar membrane (fenestra in the lateral semicircular canal) and another on the tympanic side (round window) enable an effective sound wave to reach the basilar membrane. Whether the sound wave enters the labyrinth by the new window on the vestibular side of the basilar membrane or by the round window on the tympanic side is not finally decided; though on general principles one would expect the incoming sound wave to travel along the path of least resistance.

Recently Skoog (1951) has emphasized the importance of differential sound pressure between the two windows. By loading the tympanic membrane, the hearing improved in certain cases after fenestration; this suggested that the sound wave enters the labyrinth by the new fenestra.

What happens when a window is made in the lateral semicircular canal, when both oval and round windows are freely mobile, leads to another interesting problem in sound conduction: here, instead of a movement of fluids in the vestibular labyrinth encouraging a cochlear response, almost the reverse happens, and a sound wave can result in a vestibular response.

4. The Tullio Effect

Tullio (1916) found that, by making an opening into the bony semicircular canal of a pigeon, exposure to a fairly loud sound was followed by a vestibular response. He felt that this response indicated that the vestibular labyrinth played some part in determining the direction of sound. The experimental work of Huizinga (1936) made it clear, however, that the Tullio effect was due to the creation of a new low-resistance acoustic pathway through the inner ear. Acoustic energy radiated from the stapes footplate, instead of being dissipated, as usual, through the round window, was held to be diverted through the new opening in the semicircular canal, and would thus excite its sensory end-organs.

Huizinga pointed out, as a corollary, that the Tullio effect was dependent upon an effective conductive mechanism, and in this we find the explanation of its absence following fenestration operations for otosclerosis, in which the conductive mechanism is usually grossly impeded.

These views accord well with those of Cawthorne (1949, 1955), who has attributed the Tullio effect to the presence of two mobile windows on the vestibular side of the basilar membrane: that is to say, a mobile stapes, and a window in the bony semicircular canal. In man, the Tullio effect can be so disturbing that it may be necessary to destroy the labyrinth; so care should be taken never to make a window in a bony semicircular canal when the stapes is freely mobile.

5. Organic Lesions of the Central Connexions of the Eighth Nerve System and their Localization by Surgery

Disturbances of eighth nerve function are common accompaniments of organic disorder of the central connexions of the eighth nerve system. The subject is a difficult one and still in the early stages of its development. Nevertheless, some recent advances have emphasized the importance of surgery in providing vital anatomical data. As an example of this may be quoted the investigations of Carmichael, Dix & Hallpike (1954), described elsewhere in this Bulletin[1], upon the effects of cerebral lesions on vestibular and optomotor function. In much of their material the character and localization of the lesions was accurately established by surgical exploration carried out by Mr Wylie McKissock.

In many of the patients with unilateral cerebral lesions it was possible to carry out detailed pathological studies of the affected hemispheres removed at operation. In this way it was possible to establish an illuminating functional relationship between certain areas of the cerebral cortex and disorders of vestibular and optomotor function. These, then, are some of the ways in which surgery has contributed to the problems of neuro-otology. Developments and refinements in surgery have helped to solve some problems but at the same time have served to disclose new ones—which is why neuro-otology is such an absorbing branch of medicine.

[1] See Carmichael, Dix & Hallpike, p. 146 of this number of the Bulletin.—Ed.

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PATHOLOGY, SYMPTOMATOLOGY AND DIAGNOSIS OF ORGANIC AFFECTIONS OF THE EIGHTH NERVE SYSTEM

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1 Disorders of the labyrinth
  a Ménière's disease
  b Positional nystagmus of the benign paroxysmal type
2 Disorders of the eighth nerve
  a Tumours of the eighth nerve
  b Vestibular neuronitis
3 Disorders of the central connexions of the eighth nerve within the brain-stem
4 Disorders of the cerebral hemispheres
  a Cochlear function
  b Vestibular function
  c Optomotor function
References

The organic disorders of the eighth nerve system are conveniently considered under the following headings:

i. The labyrinth.
ii. The eighth nerve.
iii. The central connexions of the eighth nerve within the brain-stem.
iv. The cerebral hemispheres.

In each of these groups the clinical signs and symptoms exhibit a distinctive pattern which depends in part upon the special pathology of the lesions themselves and in part upon their regional anatomy.

1. Disorders of the Labyrinth

The end-organs of hearing and equilibrium are closely associated within the bony labyrinth, and cochlear and vestibular function are for this reason generally deranged together by labyrinthine disease, whether it arises from infection, vascular occlusion or upset of endolymph circulation. Moreover, the clinical features of their derangement commonly exhibit a paroxysmal character dependent in part upon the properties of the end-organs, and in part upon the mechanism of the pathological processes which affect them.

Two varieties of labyrinthine disease are of particular interest to neuro-otologists: Ménière's disease, and positional nystagmus of the benign paroxysmal type.

a. Ménière's Disease

For information upon the symptomatology and natural history of Ménière's disease we need not go far beyond Ménière's own writings (1861). It is essentially a disorder of middle life, without preference for sex. Its course is benign, in so far that it does not directly shorten life, and it exhibits no association with any other form of organic disease, including allergy, outside the eighth nerve system. Partial deafness of one ear with tinnitus provides the usual clinical background upon which are superimposed the characteristic paroxysms of vertigo with nausea and vomiting. In addition to deafness, intolerance of loud sounds—with distorted perception of speech and music—are common and characteristic symptoms. Functional examination between the attacks nearly always reveals abnormalities limited to the cochlear and vestibular apparatus. Deafness of varying degree is always present. It is of the perceptive type, and loudness recruitment, often with over-recruitment, is a constant finding.

The response of the hearing defect to amplification by means of hearing aids is characteristically disappointing, and is exemplified in the speech audiogram shown in fig. 6 of the paper by Dix in this number of the Bulletin[1].

Tests of vestibular function nearly always reveal evidence of abnormality. Nystagmus is often present during the attacks, though absent between them. Positional nystagmus is extremely rare. With the caloric tests, however, abnormalities are revealed in nearly 90% of the subjects at the first examination (Cawthorne & Hallpike, 1943). Details of the results obtained in 100 cases are given in Table I.

TABLE I. RESULTS OF CALORIC TESTS IN 100 CASES OF MENIERE'S DISEASE

Caloric test findings	Number of cases
Directional preponderance	21
Canal paresis	49
Directional preponderance + canal paresis	18
Normal	12

Pathology

The development of present-day knowledge of the pathology of Ménière's disease, though spread over more than 100 years, has been achieved in a series of steps, few in number but decisive.

Flourens' experimental demonstration (1824) of the grave derangement of equilibrium resulting from labyrinthine lesions provided the starting-point for Ménière's clinical studies, which led him to the identification of the particular variety of human vertigo which has since borne his name, and which he ascribed (1861) to a lesion of the semicircular canals.

No direct evidence relating to the nature or site of the lesion thereafter became available until the description, published in 1938 by Hallpike & Cairns, of the histological findings in the labyrinths of two characteristic cases. The essential features of these findings, since widely confirmed, consisted of a dilatation, without evidence of infection or trauma, of the endolymph spaces of the cochlea and saccule, with degenerative changes in the cochlear and vestibular sense organs. The mechanism of the endolymph distension has been the

[1] See Dix, p. 119.—Ed.

subject of much discussion and investigation, centred in the main upon Guild's concept (1927) of an endolymph circulation with its source in the stria vascularis of the cochlea and its absorption area in the connective tissue surrounding the saccus endolymphaticus.

Particularly relevant are the studies of the effects of saccus extirpation (Lindsay, 1947), of sympathetic stimulation (Seymour, 1954) and of sympathetic and parasympathetic denervation of the inner ear (Rambo, Woolf & Freeman, 1953). Structural alterations induced by these procedures have either been conspicuously absent or at the most surprisingly small.

The mechanism of the endolymphatic dilatation of Ménière's disease thus remains unexplained and indeed inexplicable upon the basis of our present knowledge and ideas relating to the nature and circulation of the endolymph. New lines of inquiry are needed, and Bast's discovery (1928) of the utriculo-endolymphatic valve, and the remarkable biochemical properties of the endolymph described by Smith, Lowry & Wu (1954), may prove to be of high significance.

b. Positional Nystagmus of the Benign Paroxysmal Type

Bárány (1921) was the first to describe a peculiar clinical syndrome in which nystagmus, often accompanied by vertigo, was precipitated by certain critical orientations of the head. The studies of Nylén (1931) showed that, in a proportion of such subjects, deep-seated lesions could be demonstrated within the brain-stem or cerebellum, but no analysis was given of their precise anatomy or neurological mechanism.

In other subjects, evidence has been adduced by Gerlings (1948) and others that positional nystagmus may result, as suggested by Bárány himself, from lesions of the inner ear, and in this respect the position has been greatly clarified by the clinico-pathological studies of Dix & Hallpike (1952), who were able to show that in a large group of subjects the natural history of the disorder, its associated clinical features and, finally, the nystagmus itself, all exhibited distinctive characteristics strongly indicative of an otolith lesion.

Adults, without preference for sex and within the age-group 40–60 years, were chiefly affected, and neurological abnormalities beyond the eighth nerve system were conspicuously absent. In a significant proportion of cases, clear evidence of ear disease was present. In some this took the form of an infection, usually chronic, of the middle ear. In others, an abnormality of the caloric responses was present alone.

In some of the cases the ear lesion was unilateral, and in a very high proportion of these it was located in the ear placed undermost in the critical test position found to evoke the nystagmus. In some of the subjects head injury appeared to be the cause, while in others some evidence of focal infection was found.

The benign course of the disease, the absence of neurological abnormalities beyond the eighth nerve system, and the highly significant association with ear disease, led Dix and Hallpike to conclude that this variety of positional nystagmus, termed by them the " benign paroxysmal " type, was due to a lesion resulting from trauma, low-grade infection, or vascular occlusion affecting the otolith apparatus of the ear placed undermost in the critical test position. This conclusion they were able to substantiate by means of a histological study of the temporal bones in a characteristic case. The changes found in the otolith apparatus of the affected labyrinth are shown in Plate III, figs. A–D.

The clinical evidence of Dix and Hallpike relating the positional nystagmus to a lesion of a particular labyrinth has recently found support in the findings of Citron & Hallpike (1956) who describe two characteristic cases in which severe deafness was present in the ear placed undermost in the test position. In both, the positional nystagmus was abolished by destruction of the affected labyrinth.

Dix and Hallpike's histological evidence, too, has now been supplemented by the results of a further clinico-pathological study (Cawthorne & Hallpike, unpublished) of a subject in whom positional nystagmus of the typical benign paroxysmal type had been present for a comparatively short period. Definite pathological changes were present, localized to the macular epithelium and its associated nerve fibres of the labyrinth placed undermost in the test position.

2. Disorders of the Eighth Nerve

In this group are included two conditions of great clinical importance: tumours of the eighth nerve, and vestibular neuronitis.

a. Tumours of the Eighth Nerve

The common tumour, the neurofibroma, arising as it often does in the manner described by Crowe & Hardy (1936) within the depths of the internal auditory meatus, brings about an early loss of cochlear and vestibular function, usually accompanied by radiological evidence of bone erosion. Its development is insidious. The deafness, being unilateral, causes little disability, and is not as a rule accompanied by severe tinnitus. Loss of vestibular function, pursuing a slow and steady course, evokes no paroxysmal disturbances, and with it keep pace the processes of central compensation. As a result, this otological stage may sometimes progress to complete destruction of both cochlear and vestibular elements without any serious complaints of deafness or any vestibular symptoms apart from slight unsteadiness and momentary vertigo on walking.

The neurological stage of such a tumour begins with its emergence from the porus acusticus, and its interference with the neighbouring parts of the central nervous system. Thus, pressure upon the vestibular nuclei causes nystagmus to the opposite side; upon the descending root of the trigeminal, a loss of sensation for painful stimuli over the forehead and cornea; and upon the restiform body and cerebellum, an ataxia of the homolateral limbs with nystagmus to the affected side. At this stage, too, involvement of the facial nerve may be present on the affected side, and Edwards & Paterson (1951), in their analysis of the clinical features of these tumours, described its occurrence in 63% of their large series of cases. The otological signs and symptoms usually exhibit a distinctive pattern. As already indicated, paroxysmal attacks of vertigo are rare. Tests of vestibular function always give abnormal results. Spontaneous nystagmus is present in all cases that have reached the neurological stage, while changes in the caloric responses on the affected side occur without exception. A loss of sensitivity, a so-called canal paresis, is the commonest abnormality; this usually occurs alone, but sometimes in combination with a directional preponderance to the unaffected side. Very occasionally the latter abnormality may be present alone.

The deafness is of the perceptive type and may be of any degree. It arises chiefly from destruction of the cochlear nerve fibres and not, as in Ménière's disease, from hair-cell

degeneration. Hence, as shown by Dix, Hallpike & Hood (1948), Eby & Williams (1951) and others, loudness recruitment, so typical of Ménière's disease, is characteristically absent or incomplete. Complete loudness recruitment may occur, and has been found by Dix & Hood (1953) in 8% of their cases. This was ascribed (Dix, Hallpike & Hood, 1949) to hair-cell changes resulting from interference by the tumour with the cochlear blood supply, a view which has been supported by the finding of its disappearance following the removal of the tumour (Dix & Hood, 1953; Elliott & McKissock, 1954).

Pathology

The morbid anatomical changes in the eighth nerve and the labyrinth are well known. The peripheral cochlear and vestibular neurones within the cells of the spiral and Scarpa's ganglion undergo degeneration, usually with preservation of the hair cells of Corti's organ. On occasions, however, the labyrinthine blood supply is interfered with and changes then occur in the end-organs, sometimes advancing to necrosis of the cochlear contents.

Nearly all eighth nerve tumours pass through the otological stage described. Diagnosis is then nearly always possible by means of appropriate tests of cochlear and vestibular function. Removal of the tumours in this stage is easy, and recovery without neurological disability rapid and uneventful. Indeed, as Elliott & McKissock (1954) have shown, useful cochlear function may sometimes be preserved.

b. Vestibular Neuronitis

In organic disorders of the labyrinth or of the eighth nerve, cochlear and vestibular function are generally deranged together, with a resulting association of disequilibrium, deafness and tinnitus.

In 1952, however, Dix & Hallpike drew attention to the fact that, in a substantial proportion of subjects referred to their neuro-otological clinic on account of paroxysmal vertigo without evidence of organic disease of the central nervous system, cochlear signs and symptoms were conspicuously absent. The results of their detailed clinical analysis are summarized as follows.

Adults were found to be affected without preference for sex, and the age-groups under 30 years were more commonly affected than in Ménière's disease. In some of the cases, certain peculiarities were noted in the character of the vertigo, which might take the form of transient seizures accompanied by sensations of black-out. In others, however, severe paroxysms were absent, and the disequilibrium then took the form of feeling " off-balance " when walking. Deafness and tinnitus were conspicuous by their absence, and the most searching tests of cochlear function yielded no evidence of abnormality. Objective evidence of vestibular disorder was never wanting. In particular, abnormalities of the caloric responses, usually severe and often bilateral, were constantly present, while the galvanic responses, too, were frequently reduced. In a significant proportion of subjects the onset of the symptoms was found to be associated with some kind of infective illness, while in others examination revealed clear evidence of oro-nasal infection. As with Ménière's disease, however, middle-ear infection appeared to play a negligible role. The clinical course of the condition was essentially benign, and the vertigo usually responded rapidly to treatment of infective foci when these were present, sometimes with restoration of the caloric responses.

The pathological basis of the vestibular disorder thus identified remains to some extent a matter of conjecture. The gross defects of the caloric responses, taken in conjunction with the absence of cochlear involvement, were thought to provide strong evidence of an organic lesion of the vestibular neurones at some point central to the labyrinth. No specification was, however, possible of the particular neurone, peripheral or central, or of the neuronic elements, nerve fibres or nerve cells which were involved, and it is for this reason that Dix & Hallpike (1952), in describing the condition, introduced the comprehensive descriptive term " vestibular neuronitis ".

The view that the neuronic injury is due to the localized action of a toxin is in accord with the well-known selective vulnerability of the vestibular nervous elements to streptomycin, and it may well be that the infective processes found to occur in association with vestibular neuronitis owe their vestibulo-toxic effect to a chemical affinity of their circulating products to the streptomycin molecule.

The contribution of Hamberger & Hydén (1949) to our knowledge of the chemistry of the vestibular ganglion cells is well known, and it would seem possible that an extension of their studies—in particular relationship to the chemistry of streptomycin and of certain bacterial and other toxins of known clinical importance—will in due course throw more light upon the pathological mechanism of vestibular neuronitis.

The four conditions so far considered—Ménière's disease, positional nystagmus of the benign paroxysmal type, tumours of the eighth nerve, and vestibular neuronitis—are found to be present in a very high proportion of the subjects of organic disease of the eighth nerve system who present themselves for examination at a neuro-otological clinic. Their differential diagnosis depends upon an analysis of their characteristic symptoms and signs, which are set out in Table II.

3. Disorders of the Central Connexions of the Eighth Nerve within the Brain-Stem

Some of the neurological derangements which result from organic lesions of the brain-stem can be closely related upon the basis of clinico-pathological data to known anatomical elements. In this way, the lesions themselves can be localized, and the information so obtained may then be extended to the elucidation of the anatomical basis, less well understood, of other and coincident neurological abnormalities.

This method of study is exemplified in a recent investigation (Carmichael, Dix & Hallpike, unpublished) of certain disturbances of eighth nerve function observed to occur in association with vascular lesions of the brain-stem. The clinical material consisted of a series of adults, all in-patients at the National Hospital, Queen Square, who had sustained unilateral occlusive lesions of one or other of the arteries supplying the brain-stem. In the majority of the subjects, the posterior inferior cerebellar artery was considered upon the neurological evidence to be the affected vessel. Disturbances of vestibular function, associated in particular with one or other of the abnormalities of the caloric test responses described elsewhere in this symposium[2], were present in all but one of the subjects, accompanied in a few by disturbances of cochlear function.

Accurate localization of the lesions was possible upon the basis of the neurological findings related to the long tracts

[2] See Cawthorne, Dix, Hallpike & Hood, p. 131 of this number of the Bulletin. —ED.

TABLE II. SYMPTOMS AND SIGNS OF ORGANIC DISEASE OF THE EIGHTH NERVE SYSTEM

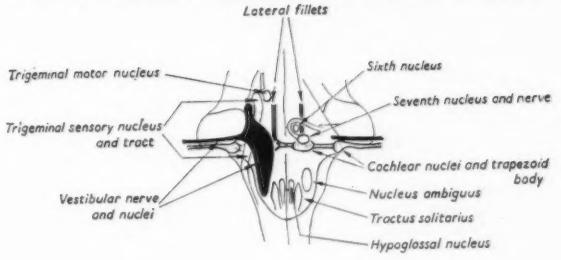
Disease	Character of vertigo	Course of vertigo	Relationship of vertigo to head position	Caloric responses	Deafness	Tinnitus	Character of deafness	Response to amplification	Loudness recruitment	Pathology
Ménière's disease	Paroxysmal, with nausea and vomiting	Attacks often severe at onset. Long periods of freedom common	Absent	Abnormal responses occur in 90%	Always present	Always present. Often severe	Variable. Sounds and voices distorted	Poor	Always present. Over-recruitment may occur	Distension of endolymph system. Degenerative changes present in end-organs. Nerve fibres and ganglion cells not affected
Tumours of cerebello-pontine angle	Paroxysms rare. Persistent imbalance usual	Slight in early stages. Thereafter progressive	Rare	Abnormal responses occur in 100%	Always present	Always present. Not often severe	Progressive	May be good	Usually absent or incomplete	Degeneration of fibres of eighth nerve and associated ganglion cells. Degeneration of end-organs not usual but may result from derangement of vascular supply
Vestibular neuronitis	Black-outs or drop seizures. Sometimes persistent imbalance	Attacks frequent during active stages. Recovery or long remissions usual	Absent	Abnormal responses occur in 100%. Often bilateral	Cochlear symptoms and signs absent					Unknown. ? Toxic degeneration of vestibular neurones
Positional nystagmus of benign paroxysmal type	Paroxysmal	Attacks frequent during active stages. Recovery or long remissions usual	Always present	Often normal	Cochlear symptoms and signs present only when condition is associated with ear disease, usually middle-ear infection					Degeneration of maculae of otolith organs. Character of tissue changes suggestive of trauma, infection or vascular upset

and cranial nerves other than the eighth, and the information so obtained made it possible to explain the various disturbances of eighth nerve function upon the basis of a functional differentiation within its intramedullary elements.

The anatomical relations of the cochlear and vestibular elements in the brain-stem are shown in fig. 1, adapted from Spalteholz (1903). The territory within the brain-stem supplied by the posterior inferior cerebellar artery rarely extends higher than the level of entry of the eighth nerves (J. G. Greenfield, personal communication). This point is important, since the affected zone of the brain-stem is thereby limited in most of our subjects to levels below that shown in fig. 1, at which the cochlear fibres pass in, join the trapezoid body and travel upwards mainly upon the opposite side of the brain-stem. By contrast with the vestibular elements, there are no cochlear elements below the level of entry of the eighth nerve.

The vestibular nerve enters the brain-stem ventral to the restiform body on its way to the ipsilateral vestibular nuclei. In their path the vestibular nerve fibres are intimately related to the restiform body, the descending nucleus and tract of the fifth nerve, the cochlear nerve fibres, the sixth nerve nucleus and the seventh nerve nucleus as it lies dorsal to the trapezoid body. The vestibular nuclei extend both upwards

FIG. 1. ANATOMICAL RELATIONS OF THE COCHLEAR AND VESTIBULAR ELEMENTS IN THE BRAIN-STEM



and downwards from this level: the medial nucleus being prolonged upwards as von Bechterew's nucleus to the level of the motor nucleus of the fifth nerve, while below it extends to the level of the hypoglossal nucleus.

These differing neuro-anatomical associations of the various levels of the vestibular nuclei thus provide a valuable guide to the localization of lesions within the nuclei themselves. Thus, lesions of the nuclei above the entry of the eighth nerve will tend to be associated with deafness, motor weakness of the face, diplopia due to abducens palsy, and sensory disturbances including loss of touch perception over the whole territory of the ipsilateral fifth nerve.

By contrast, lesions of the vestibular nuclei below the level of entry of the eighth nerve will be unaccompanied by deafness. They are likely to involve the spinothalamic tract, the restiform body, the descending nucleus and tract of the fifth nerve, and the nucleus ambiguus, and will so give rise to disturbance of painful perception over the opposite half of the body, incoordination of the ipsilateral limbs, disturbance of pain perception over the first division of the ipsilateral fifth nerve, and paresis of the ipsilateral vocal cord and half of the soft palate. Lower still there may be involvement of the cuneate nucleus and the ninth and tenth nuclei, with sensory changes over the ipsilateral upper limb, hiccough and disturbance of taste.

All but one of our subjects exhibited abnormalities of the caloric responses. In the majority, directional preponderance alone was present to the opposite side without any evidence of canal paresis. In a few others, however, canal paresis was present alone. These differences appeared to be related quite simply to the level of the lesion. Thus, directional preponderance was present when the lesions affecting the vestibular nuclei lay below the level of entry of the eighth nerve. With lesions above this level, however, canal paresis appeared to be the rule.

As fully discussed elsewhere[3], these two abnormalities of the caloric responses may be encountered, either separately or in combination, in association with any of the organic disorders of the labyrinth and eighth nerve. Cawthorne, Fitzgerald & Hallpike (1942) have argued that canal paresis is due to a lesion of the sensory mechanism of the external canal or of its associated vestibular nerve fibres. Directional preponderance, however, was attributed to organic disorder of some other sense organ or organs within the labyrinth, which are concerned with the maintenance of tonus or of the vestibular nerve fibres which are associated therewith.

This functional distinction between the two nervous mechanisms concerned appears to be maintained when their nervous pathways are followed into the vestibular nuclei. Thus, the tonus elements are found to be linked with those parts of the nuclei, in particular the lower part of the medial nucleus, which lie below the point of entry of the eighth nerve, while the canal elements are linked with those parts of the nuclei which lie above this level.

4. Disorders of the Cerebral Hemispheres

The effects of cerebral lesions upon the eighth nerve system will be considered under the headings of cochlear and vestibular function.

[3] See Cawthorne, Dix, Hallpike & Hood, p. 131 of this number of the Bulletin. —Ed.

a. Cochlear Function

In man, the organization of cochlear function within the cerebral hemispheres is generally assumed to follow the pattern outlined by the results of anatomical and electro-physiological studies in other mammalian types. The subject has been fully reviewed by Rosenzweig (1951) and Galambos (1954).

The reception areas lie on the upper surface of the temporal lobes, and each is predominantly activated from the opposite cochlea. The removal of one temporal lobe, however, is found to produce a negligible effect upon hearing as judged by threshold tests, a result which is in good accord with the clinical reports of Dandy (1930) and Gardner (1932) upon the effects in man of unilateral hemispherectomy, and has since been substantiated by the detailed pure-tone audiometric studies carried out by Carmichael, Dix & Hallpike (unpublished) in patients subjected to hemispherectomy for epilepsy associated with infantile hemiplegia. In none of these was it possible to demonstrate significant abnormalities of hearing, either before or after the operation.

More recently, Bocca, Calearo, Cassineri & Migliavacca (1955) have obtained positive evidence of auditory dysfunction in cerebral disease by means of tests involving a high degree of auditory discrimination, and were able to demonstrate a slight but significant loss of intelligibility when the stimulus—distorted speech—was applied to the ear opposite to a unilateral temporal lobe lesion. Bocca's investigations provide some promise that the auditory manifestations of cerebral disease will within the near future be brought within the scope of practical neuro-otology.

b. Vestibular Function

Beyond the association of a high incidence of vertigo with organic lesions of the temporal lobe, symptomatology provides no guide to the organization of vestibular function within the human cerebrum.

Objective methods of investigation, both in man and experimental animals, have, however, provided us with a body of information which is now considerable. Thus, Spiegel (1932), by means of electrical studies of the strychnine-treated cortex of the cat, was able to show a marked localization within the temporal lobe of the cortical response to vestibular stimulation. In man, the association of the temporal lobe with vestibular function has now been established by the results of a series of investigations beginning in 1923 with the work of Dusser de Barenne & de Kleyn. These workers were able to show that removal of one hemisphere in the rabbit brought about a certain abnormality in the nystagmic response to vestibular stimulation, to which the term "directional preponderance" has since been applied. Thus, the nystagmic reactions, having their rapid components directed to the side of the lesion, were found to be increased, while the opposite reactions were diminished.

In addition, de Barenne & de Kleyn (1923) were able to demonstrate a similar derangement in a few subjects suffering from extensive unilateral cerebral lesions, and these findings have been confirmed and extended as the result of systematic studies which have since been carried out at the National Hospital, Queen Square (Fitzgerald & Hallpike, 1942; Carmichael, Dix & Hallpike, 1954). The clinical material used for the latter of these two investigations consisted of two groups of subjects:

Group 1. Widespread unilateral lesions of one hemisphere associated with hemiplegia and epilepsy. In many of these,

the nature and extent of the pathological process was established by direct investigation of the hemisphere removed by operation. In all, there was found a marked directional preponderance—to the side of the affected hemisphere—of the nystagmic responses to caloric stimulation.

Group 2. Localized lesions of one hemisphere. The lesions were scattered throughout the hemisphere in the manner shown in figs. 2 and 3. Directional preponderance of caloric nystagmus was found to be absent with all of the lesions shown in fig. 2; it was present with all of the lesions shown in fig. 3, and the directional control of vestibular nystagmus was thus shown to be dependent upon centres lying in the posterior part of the temporal lobes. The authors stressed the fact that vestibular nystagmus is primarily dependent upon the vestibular nuclei with its associated connexions with the eye muscle nuclei and labyrinths, and can be readily induced in the absence of the cerebral hemispheres. They also stressed that each set of vestibular nuclei, right and left, is concerned with vestibular nystagmus occurring in the homolateral direction.

For these reasons they concluded that the effect of the temporal lobes upon vestibular nystagmus is a modifying one, exerted by means of corticifugal fibres passing to the vestibular nuclei. Thus, each temporal lobe was believed to be associated through these fibres with the vestibular nuclei on one or other side of the brain-stem. Hence, destruction of one temporal lobe would result in a corresponding directional preponderance of induced nystagmus.

c. Optomotor Function

As with vestibular nystagmus, optomotor or optokinetic nystagmus is essentially a brain-stem reflex (Smith & Bridgman, 1943) depending upon direct nervous pathways from the optic tracts to the superior colliculi and eye muscle nuclei. If these are preserved, then optokinetic nystagmus may be produced following removal of the hemispheres.

The brain-stem mechanism of optokinetic nystagmus has long been thought to be closely related to the vestibular mechanisms and, indeed, Ohm (1936) has claimed that the vestibular nuclei themselves provide an essential link in the optokinetic mechanism. This view has since been controverted by the demonstration (Dix, Hallpike & Harrison, 1949) that, in cases of severe streptomycin intoxication, the optokinetic responses remain entirely unaffected, in spite of the

FIG. 2. LOCALIZED UNILATERAL CEREBRAL LESIONS, WITH ABSENCE OF DIRECTIONAL PREPONDERANCE OF CALORIC NYSTAGMUS

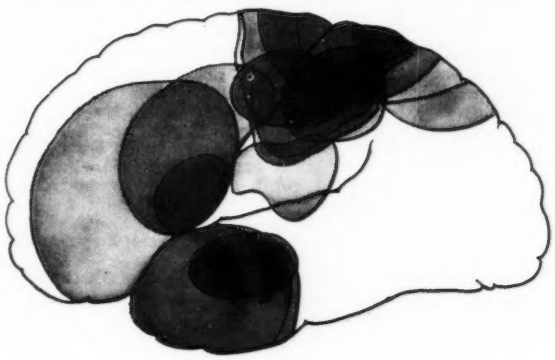
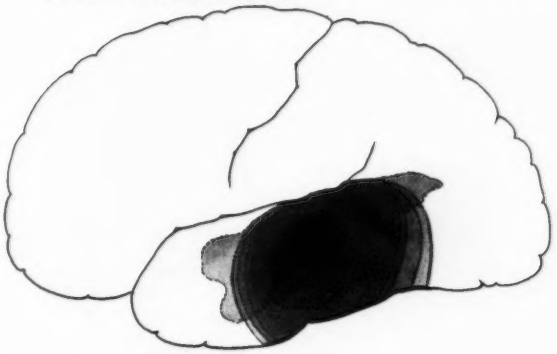


FIG. 3. LOCALIZED UNILATERAL CEREBRAL LESIONS, WITH DIRECTIONAL PREPONDERANCE OF CALORIC NYSTAGMUS TO THE SIDE OF THE AFFECTED HEMISPHERE



abolition of both caloric and galvanic responses involving, in all probability, some organic derangement of the vestibular nuclei. More recently, Blackwood, Carmichael & Hallpike (unpublished) have described the preservation of optokinetic nystagmus in a case of sensory neuronopathy with severe degenerative changes in the vestibular nuclei themselves.

Thus, it seems likely that the supratentorial connexions of the optokinetic mechanisms pass to the eye muscles via the superior colliculi by pathways that are entirely separate from the vestibular nuclei and may possibly involve gaze centres that are independent of those that subserve vestibular nystagmus. The arrangement postulated by Dix, Hallpike and Harrison is shown in fig. 4.

In neuro-otological practice, lesions of the brain-stem involving the posterior longitudinal bundle are commonly found to be associated with derangement of optokinetic nystagmus. In addition, certain unilateral cerebral lesions may also bring

FIG. 4. BRAIN-STEM MECHANISMS OF OPTOKINETIC AND VESTIBULAR NYSTAGMUS (arrangement postulated by Dix, Hallpike & Harrison, 1949)

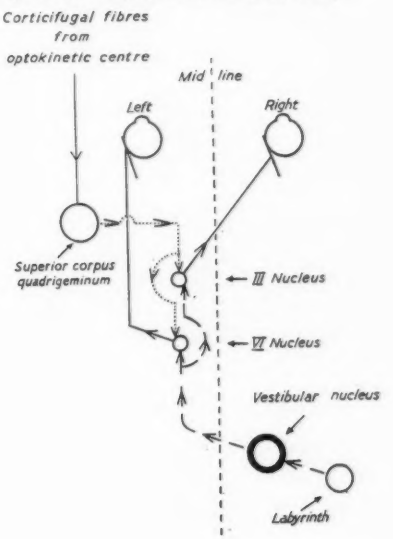
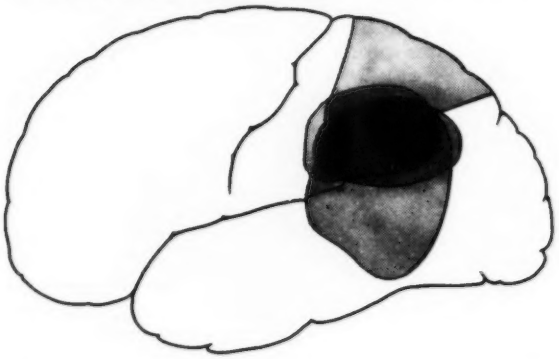


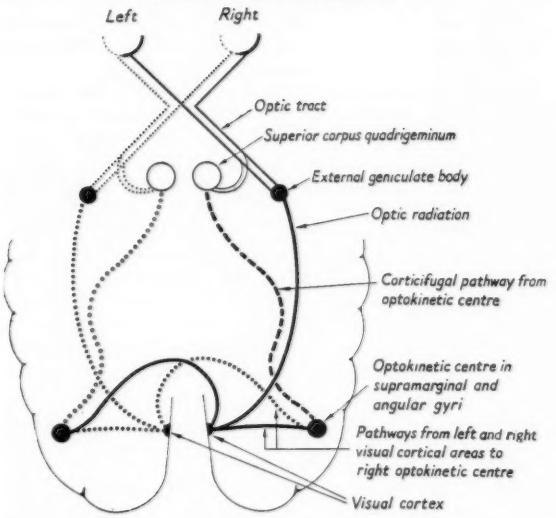
FIG. 5. LOCALIZED UNILATERAL CEREBRAL LESIONS, WITH DIRECTIONAL PREPONDERANCE OF OPTOKINETIC NYSTAGMUS TO THE SIDE OF THE AFFECTED HEMISPHERE



about a diminution or abolition of optokinetic nystagmus having its rapid component to the side opposite to the lesions: in effect, a directional preponderance to the side of the lesion. The neurological mechanism of this has been studied by Fox & Holmes (1926), Rademaker & ter Braak (1948) and more recently by Carmichael *et al.* (1954). The findings of the latter are summarized as follows: (i) no optokinetic abnormality was encountered in association with any of the unilateral cerebral lesions shown in figs. 2 and 3; (ii) directional preponderance of optokinetic nystagmus to the side of the affected hemisphere was, however, associated with the unilateral cerebral lesions shown in fig. 5.

These findings were considered to provide strong evidence that the cerebral mechanism responsible for the directional control of optokinetic nystagmus was situated in the supramarginal and angular gyri. In fig. 6 is shown a schematic reconstruction of the cerebral mechanism of optokinetic nystagmus in man, based upon clinical data and the results of animal experiments carried out by Smith & Bridgman (1943).

FIG. 6. SUGGESTED SCHEMA OF CEREBRAL OPTOKINETIC MECHANISMS



Each of the optokinetic centres is connected with the entire retinas of both eyes via both occipital cortical areas. Efferent fibres from each optokinetic centre then pass forward, at first in association with the optic radiation, and thereafter join the brain-stem optokinetic mechanism at or near to the homolateral superior colliculus. A unilateral lesion involving the optokinetic centre, the superior colliculus or the intervening nervous pathway will thus bring about a homolateral preponderance of optokinetic nystagmus.

ACKNOWLEDGEMENTS

We acknowledge with thanks the permission of the Royal Society of Medicine to reproduce Plate III, figs. A–D, and of the editor of *Brain*, for permission to reproduce figs. 2–5.

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ORGANIC AFFECTIONS OF THE EIGHTH NERVE SYSTEM

(FIGS. A—D)

E. A. Carmichael, M. R. Dix & C. S. Hallpike

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FIG. B (magnification × 100)

FIG. A (magnification × 16)

FIGS. A, B. Histological structure of the normal human utricular macula

FIG. D (magnification × 100)

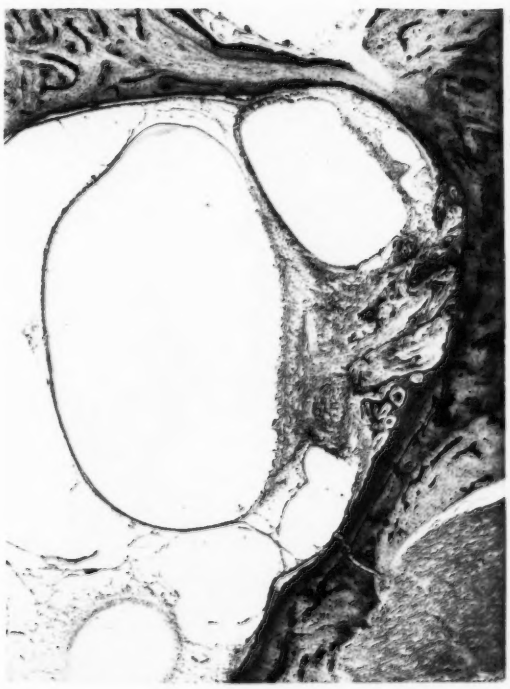


FIG. C (magnification × 16)

FIGS. C, D. Corresponding views of the utricular macula in a subject with positional nystagmus of benign paroxysmal type

PLATE IV (FIGS. A—H)

HEREDITARY LESIONS OF THE LABYRINTH IN THE MOUSE

(FIGS. A—H)

Hans Grüneberg

p. 153

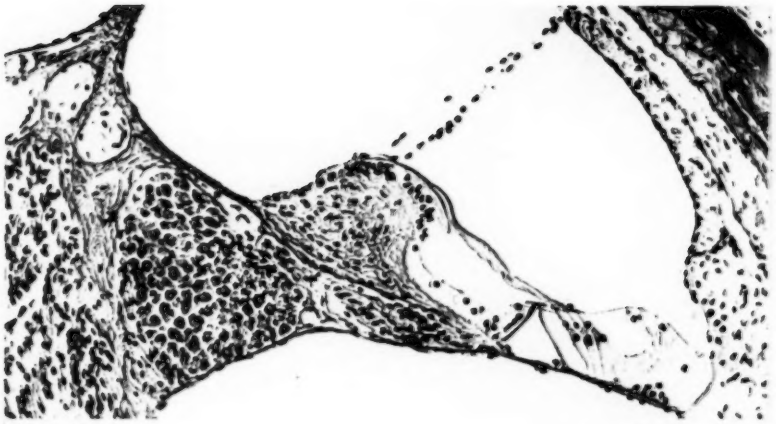


FIG. A (magnification x 208)

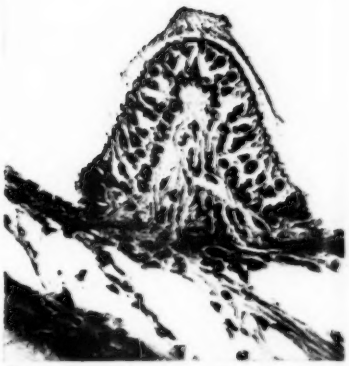


FIG. C (magnification x 335)

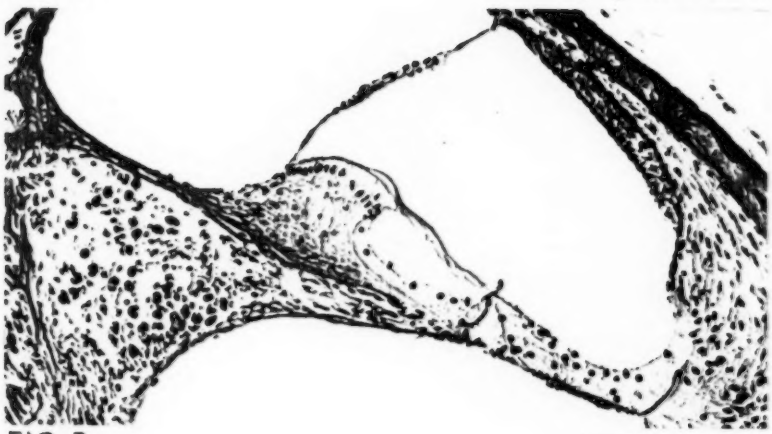


FIG. B (magnification x 208)

FIG. D (magnification x 335)

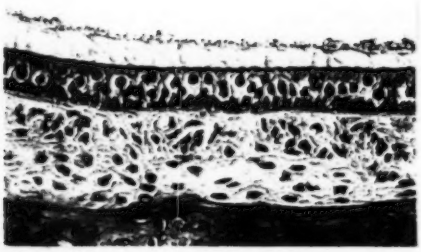


FIG. E (magnification x 335)

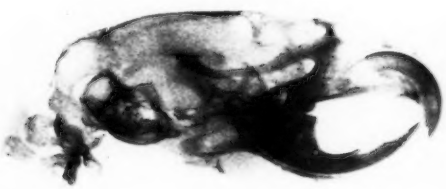


FIG. G

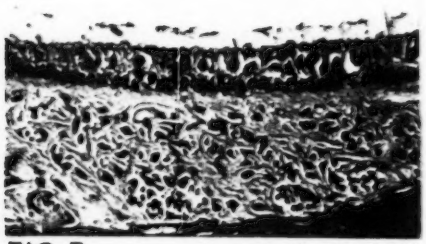


FIG. F (magnification x 335)

FIG. H

HEREDITARY LESIONS OF THE LABYRINTH IN THE MOUSE

HANS GRÜNEBERG M.D. D.Sc. F.R.S.

Medical Research Council
Group for Experimental Research in Inherited Diseases
University College, London

1 The waltzer-shaker group of mutants
2 Mutants with degenerative changes in the labyrinth
3 Mutants with morphogenetic defects of the labyrinth
4 Shaker with syndactylism
5 Uncomplicated deafness
6 Defect of the otoliths
7 Conclusions
References

The study of the pathology of labyrinthine disorders in man has to contend with considerable technical difficulties and with a scarcity of material, particularly of the initial stages. Moreover, one and the same clinical label may cover several aetiological mechanisms. Under these circumstances, it is fortunate that many comparable inherited disorders of the labyrinth are known in the laboratory mouse: no less than fifteen genetically distinct conditions have now been studied histologically, and several others remain to be examined. One gene, each affecting the labyrinth, has been described in the rat, the guinea-pig, the rabbit, the cat and the dog, but not all of these have so far been adequately studied. The present report will deal with the mouse only and will include only those conditions for which pathological or embryological data are available.

1. The Waltzer-Shaker Group of Mutants

The prototype of inherited disorders of the labyrinth in the mouse is the so-called Japanese waltzing mouse which reached European and American laboratories from the Far East towards the end of the nineteenth century and which has been extensively studied by physiologists and otologists. However, the resulting large literature (reviewed by Grüneberg, 1952) is full of contradictions and is most unsatisfactory. Apart from inadequacies of histological technique, this is mainly due to two reasons. It was not realized by the early workers that the waltzing syndrome can be brought about by different genes with different pathological mechanisms; and so the early papers clearly do not all refer to the same condition. Secondly, it was not realized that among clinically normal (i.e., non-waltzing) mice, anomalies of the cochlea may be found; and so some of the "normal controls" may not, in fact, have been normal. The whole group has been reduced to a state of order comparatively recently by the use of improved histological techniques and of genetically analysed strains of mice each of which carries a single condition only.

Most of the conditions to be described presently have been discovered through the abnormal behaviour of the animals. This is the waltzer-shaker syndrome mentioned above which, when fully developed, consists of the triad of head shaking, circular movements and deafness; an additional feature usually encountered is inability to swim. The head shaking is present in all mutants of this group and is usually in the sagittal plane, the head being thrown backwards repeatedly; but several mutants of this group shake their heads mainly from side to side. The circular movements vary in intensity both within and between strains. Young mice in the nest do not react to sounds; a reaction (pinna reflex) can first be elicited at the age of about a fortnight. Eight of the 12 mutants of this group never show any response to auditory stimulation; one mutant can hear for a short period during adolescence but becomes deaf before reaching sexual maturity; and in three of the mutants the sense of hearing is normal. Nine of the mutants show no other abnormalities and thus can be recognized when they are alive by their abnormal behaviour only; but in three cases there are additional features which will be mentioned.

The 12 mutants of the waltzer-shaker group fall into two sharply distinct categories of six conditions each. In the first of these, the gross structure of the labyrinth is normal; regressive and degenerative changes in the neuro-epithelia and ganglion cells supervene some time during early growth and are usually progressive. The second group includes gross morphogenetic anomalies of the labyrinth which arise in early stages of embryonic development, but there is little or no subsequent degeneration of the specific sense organs so far as these have been formed.

PLATE IV. HEREDITARY LESIONS OF THE LABYRINTH IN THE MOUSE

FIGS. A–F. Abnormalities in the cochlea, the crista ampullaris and the saccular macula of Varitint-waddler (*Va*) (77-day-old litter mates)

FIGS. A, C and E: normal mouse

FIG. B: *Va*/+ heterozygote

FIGS. D, F: *Va*/*Va* homozygote

Note the absence of hair cells in Corti's organ together with the disorganization of the supporting cells and neighbouring cell groups, and the absence of the outer tunnel of Corti; the reduced number of cells in the ganglion spirale; and the reduced size of the stria vascularis. In the crista ampullaris as well as in the macula, some of the hair cells have retracted from the surface and become more spherical; their number is reduced and they are in places separated from each other by the cytoplasm of supporting cells.
For histological technique, see Deol (1954).

FIGS. G, H. Radiographs (enlarged) of the halved heads of a normal (fig. G) and a fidget mouse (fig. H)

Note the absence of the superior semicircular canal in the latter; the absence of the other canals is not visible in this plane.

2. Mutants with Degenerative Changes in the Labyrinth

These mutants are:

waltzer (symbol *v*)
shaker-1 (symbol *sh*-1; Lord & Gates, 1929)
shaker-2 (symbol *sh*-2; Dobrovolskaia-Zavadskaia, 1928)
jerker (symbol *je*; Grüneberg, Burnett & Snell, 1941)
pirouette (symbol *pi*; Woolley & Dickie, 1945)
Varitint-waddler (symbol *Va*; Cloudman & Bunker, 1945)

The first five of these are recessive and have no features in addition to the complete waltzer-shaker syndrome; i.e., they shake their heads in the sagittal plane, they circle to a varying extent and they are completely deaf (except that *sh*-1 can hear for a period which varies between a few days and a few weeks). Clinically they are so similar to each other that a trained observer who has worked with them all would hesitate to attach a genetical label to an isolated mouse of this group. Statistically, there seem to be differences between mutants; for instance, in the direction of circling and in the ability or otherwise to reverse the "waltz". However, as these mutants have not yet been compared on a uniform (isogenic) background, it is not known whether these differences are due to the main genes, or to other genes in respect of which the strains differ. The behaviour anomalies can usually be recognized at the age of 12–14 days or a little earlier. Varitint-waddler is semi-dominant, and in addition to its abnormal behaviour it shows a characteristic combination of spotting and fur colour dilution by means of which affected animals can be identified in the nest at the age of about three days. The *Va*/*Va* homozygotes are more severely affected than the *Va*/+ heterozygotes; they are lighter in colour, their general viability is much reduced, and they are liable to epileptiform seizures in addition to the usual syndrome.

The pathology of the labyrinth of waltzer (*v*) has been studied by van Lennep (1910) and others, and that of shaker-1 (*sh*-1) by Grüneberg, Hallpike & Ledoux (1940). More recently, Deol (1954, 1956b) has investigated the remaining four members of this group in considerable detail and has re-investigated *v* and *sh*-1; the following description will be based almost entirely on his work. The six genes of this group resemble each other not only clinically, but also pathologically. All of them affect both the cochlea and the vestibular apparatus. Whereas the cochlear manifestations are very similar, the vestibular lesions vary a good deal from one mutant to another. At birth, the labyrinth of the mouse is still very immature, and the histological differentiation, particularly of the organ of Corti, largely happens during the first fortnight after birth. With a few possible exceptions which will be mentioned below, the labyrinth of all these mutants appears to be quite normal at birth. Abnormalities make their appearance as the histological differentiation approaches maturity, or at a later stage. The time of onset of the various anomalies as mentioned below refers to clearly demonstrable defects; it is possible and indeed probable that, with improved and more sensitive histological methods, some of the anomalies may be detectable at earlier stages. For that reason, not too much importance should be attached to statements that defect A appears in the second week and defect B in the third; such a small difference in timing may well vanish or even be reversed by the use of more refined methods.

The cochlea of all six mutants undergoes a degeneration of the organ of Corti, of the spiral ganglion and of the stria vascularis (see Plate IV, figs. A, B). A transitory anomaly of the membrana tectoria during the second week is marked in *sh*-2, *je* and *Va*, but slight or absent in the other three mutants; for details of the latter, the reader may be referred to Deol (1954).

Corti's organ in all six mutants shows normal histological differentiation up to the twelfth day, when histogenesis is virtually complete. Thereafter, the hair cells gradually shrink and their nuclei become pyknotic; the nuclei migrate upwards away from the basilar membrane and disappear on reaching the reticular lamina. The supporting cells dedifferentiate and merge with neighbouring groups of cells; they may ultimately invade the tunnel of Corti. The membrana tectoria is curled up and not in contact with the hair cells. In *sh*-1 mice which can hear for a while, the degeneration of Corti's organ is similar, but does not happen until the mouse is 6–8 weeks old. Degeneration tends to progress with age, but sometimes the final state is reached quickly, and in other cases the process is arrested.

The spiral ganglion begins to degenerate during the second to fourth week (except in *sh*-1 where this does not happen until the age of two months). The degeneration of the ganglion seems to follow in time the visible changes in the organ of Corti. At first the amount of cytoplasm round the nuclei is reduced and the cells look more rounded; this gives the ganglion as a whole a more open appearance. Later the nuclei themselves degenerate and many disappear, though some always remain in Rosenthal's canal. Degeneration starts in the basal half-turn of the cochlea and proceeds upwards. The rate of degeneration may differ considerably from mutant to mutant; it is more rapid in *Va*/+ than in *je*, but ultimately about the same degree of degeneration is reached. In *sh*-2, by contrast, there is little degeneration during the first 100 days and it does not become as extreme as in the other two mutants.

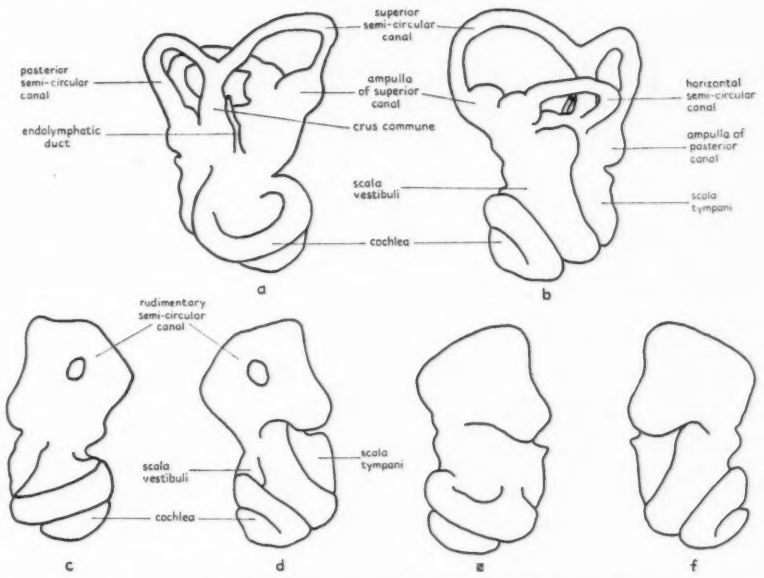
According to Deol (1954, 1956b), visible degeneration of the stria vascularis occurs later than the degeneration of the organ of Corti; it involves at first histological abnormalities and later the size of the organ as a whole. The onset is early (third week) in *Va*/+, *je* and *sh*-2, but only after two months in *v*, *sh*-1 and *pi*. The stria may be grossly abnormal in some parts of the cochlea but more nearly normal in others.

Of the vestibular lesions, the only one which is common to all six mutants is a degeneration of the saccular macula (Plate IV, figs. E, F); slight anomalies of this macula seem to be present at birth in *sh*-2 and *je*; in the others, regressive changes do not become manifest until the second to sixth week after birth, depending on the mutant involved. Similar though slighter changes in the utricular macula occur in *je*, but not in any of the other mutants. The anomaly starts with loss of hairs from the hair cells which then retract from the surface and become rounded instead of being columnar; these cells gradually disappear until sometimes little but the supporting cells of the maculae is left; in such cases the otolith membrane is disorganized and occasionally, in very old mice, membrane and otoliths may disappear. Somewhat similar changes also occur in the cristae ampullares of *Va* (after the first month; Plate IV, figs. C, D) and of *pi* (after the eighth month or so). The vestibular ganglion is affected in all mutants except (?) in *je* and *v*; there is a reduction of the mean size of the ganglionic cells, whereas their total number seems to be little if at all affected. The vestibular ganglion is affected fairly early in life in certain mutants, but only very late in others.

The causal relationships between the various anomalies are not yet clear. The lesions of the scala media complex always appear together and thus obviously form some kind of causal unit; the degeneration of the spiral ganglion is presumably a consequence of the degeneration of Corti's organ. How these lesions link up with the changes in the stria vascularis is not clear. The hypothesis put forward by earlier authors that the degeneration of Corti's organ may be secondary to the changes in the stria vascularis—the source of endolymph—is no longer tenable, as similar degenerations are now known to occur in the maculae and other structures that have a blood supply of their own and thus presumably do not depend for their nutrition on the endolymph.

A peculiar interaction between the genes for *v* and *sh*-1 has been discovered by Lord & Gates (1929) and more recently confirmed by Deol (1956b). Whereas the single heterozygotes +/*v* and +/*sh*-1 are completely normal, mice which carry both genes in single dose (+/*v*; +/*sh*-1)—though they do not shake their heads or circle—become deaf in middle life. In the labyrinths of such double heterozygotes, Deol (1956b) observed changes in the scala media complex similar to those described above. However, these changes are slight and appear very late in life—they do not become demonstrable until the mouse has been clinically deaf for quite a while. Whether defective function precedes defective structure in this case must remain uncertain; perhaps more refined histological techniques might show that structure and function become defective at the same time. Whether other genes of this group show similar interactions in double heterozygotes is not yet known.

FIG. 1. MEMBRANOUS LABYRINTHS OF NORMAL AND OF FIDGET MICE



a, b: internal and external views, respectively, of left labyrinth of normal mouse

c, d: internal and external views, respectively, of left labyrinth of fidget mouse, with rudimentary superior semicircular canal

e, f: internal and external views, respectively, of right labyrinth of fidget mouse, without any semicircular canals
(The endolymphatic ducts of the labyrinths of the fidget mice are accidentally missing from these preparations.)

3. Mutants with Morphogenetic Defects of the Labyrinth

These mutants are:

shaker-short (symbol *st*; Dunn, 1934)
kreisler (symbol *kr*; Hertwig, 1942)
fidget (symbol *fi*; Grüneberg, 1943)
dreher (symbol *dr*; Falconer & Sierts-Roth, 1951)
Twirler (symbol *Tw*; Dr Mary F. Lyon, unpublished work)
Zigzag (symbol *Zg*; Dr Mary F. Lyon, unpublished work)

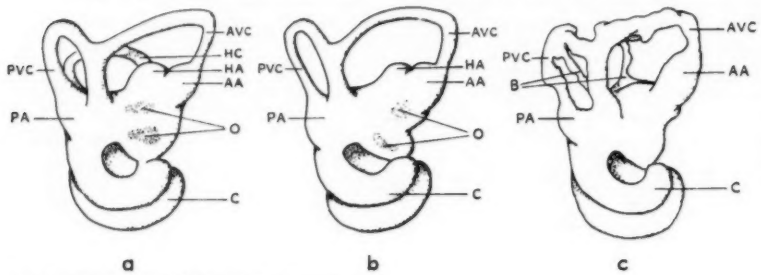
All these show gross defects of the labyrinth which must have arisen round about the ninth to twelfth day of embryonic life; for the first three or four of them, this has been demonstrated by embryological studies; in the remaining cases, this conclusion rests so far on the appearance of the labyrinth in later stages.

Shaker-short. This recessive gene (now extinct) combines reduction of the tail with cerebral hernias and profound defects of the labyrinth (Bonnevie, 1936). The inner ear is represented by an oval laterally compressed vesicle which lacks an endolymphatic duct, has no semicircular canals and no division of the median part into separate chambers. The ventral part of the vesicle shows, however, a trace of spiral curving which reminds one of a cochlea, and along the inner side of this spiral there is a structure which seems to represent a rudimentary organ of Corti. The behaviour of this mutant was more erratic than that found in the conditions described above; it was, of course, completely deaf, and its viability was greatly reduced.

Kreisler. In this recessive mutant, the ear vesicles seem to be formed too far away from the rhombencephalon (Hertwig, 1944). The endolymphatic duct is absent, there is no sacculus, and the semicircular canals are often imperfect; the ductus cochlearis remains a wide slightly curved sac and does not form a spiralized cochlea; there is no organ of Corti and no spiral ganglion. The ductus endolymphaticus is the normal drainage canal for the endolymph; presumably on account of its absence, the pressure of the endolymph is greatly increased; except for the perilymphatic spaces, the only place which can yield to this pressure is the internal acoustic meatus and, through this pathway, cysts which are often quite large evaginate into the subarachnoid space. The behaviour of this mutant is like that of the first group, i.e., dorsal head shaking, circling and deafness.

Fidget. This recessive mutant which has recently been studied by Truslove (1956) combines labyrinthine with ocular lesions (absence of the lacrimal glands, corneal ulcers, etc.) and various skeletal anomalies (fossa acetabuli, tendency to polydactylism, etc.). The labyrinthine anomalies are confined

FIG. 2. OUTLINES OF THE BONY LABYRINTH OF THE LEFT EAR OF A NORMAL MOUSE, A ZIGZAG, AND A TWIRLER



a: bony labyrinth of left ear of normal mouse

b: bony labyrinth of left ear of a Zigzag
(The ear is normal except for the absence of the horizontal canal.)

c: bony labyrinth of left ear of a Twirler
(The otoliths and horizontal canal are lacking, and both vertical canals are uneven in outline and have branches running to the utriculus.)

AA: anterior ampulla — HA: horizontal ampulla
AVC: anterior vertical canal — HC: horizontal canal
B: branch of vertical canal — O: otoliths
C: cochlea — PA: posterior ampulla
PVC: posterior vertical canal

to the vestibular apparatus (for a comparison of the labyrinth of a normal and a fidget mouse, see Plate IV, figs. G, H; and fig. 1). The horizontal semicircular canal and its crista ampullaris are missing; the superior and posterior canals remain rudimentary, but the associated sense organs are normal. The cochlea is completely normal, and the animals can hear. The head shaking is mainly from side to side, and there is some circling in most fidgets.

Dreher. Only preliminary data on this recessive gene have so far been published (Hertwig, 1951). The ductus endolymphaticus is absent or reduced, sacculus and utriculus are not separate from each other, and there are considerable defects of the semicircular canals. The cochlea has one-and-a-half turns, as in a normal mouse. In post-embryonic life, the walls of the membranous labyrinth are distended at the expense of the perilymphatic spaces as in *kr* (see above), though cysts are small or absent. Clinically, there is head shaking, very vigorous circling and deafness.

Twirler. This new gene (Dr Mary F. Lyon, unpublished work) is semi-dominant: "Heterozygotes show circling, combined with horizontal and in a few cases also vertical head-shaking, and fail to give normal reflex responses to change of position. Most Twirlers react normally to sound. The abnormal behaviour can be ascribed to defects of the inner ear, including absence of otoliths and reduction and malformation of canals. In almost all Twirlers all otoliths are absent, but a few possess reduced otoliths. The horizontal canals are reduced and often absent and the horizontal crista ampullaris is malformed. Defects of the vertical canals vary from mild unevenness of outline to outgrowing projections, and thence to actual branching of the canals. The cristae appear normal." (See fig. 2c and cf. fig. 2a, which shows the labyrinth of a normal mouse). The homozygotes are inviable; they are probably represented by a group of animals with harelip and cleft palate.

Zigzag. According to a personal communication by Dr Mary F. Lyon, "... affected animals walk with a zigzag motion of the head, make frequent changes of direction, and often end a journey with a circling movement. Some circle repeatedly. This behaviour is attributable to absence of one or both horizontal canals from the inner ear. The otoliths, vertical canals and even the horizontal ampullae appear normal but the horizontal canals themselves are lacking." (See fig. 2b.) The sense of hearing is normal. The mode of inheritance is not yet entirely clear.

4. Shaker with Syndactylism

This recessive mutant (symbol *sy*; Hertwig, 1942) differs from the typical members of the waltzer-shaker group mainly in its pathology. Affected animals usually die young and never reach sexual maturity. They can be identified even as embryos by the associated foot anomalies. According to Hertwig (1951), the membranous labyrinth of new-born *sy* mice is normal or nearly so. From about the third day onwards, there is a progressive atrophy of the membranous labyrinth which leads to a narrowing of the semicircular canals, of the sacculus and utriculus and of the scala media of the cochlea; Reissner's membrane collapses and is in contact with the membrana tectoria, and a narrow lumen remains only in the neighbourhood of the stria vascularis; later, that structure and Corti's organ also degenerate. The shrinkage of the membranous labyrinth may be the expression of an underproduction of endolymph, though there is no obvious reason why that should be so.

5. Uncomplicated Deafness

Ten out of the 13 mutants described above are deaf. This was discovered because mutants with obvious labyrinthine disturbances are of course tested for their sense of hearing. Deafness without locomotor disturbances will be discovered only by systematic hearing tests of normal mice. One recessive gene of this kind has recently come to light in a stock of mice in which it had probably been present unrecognized for a long time (Deol, 1956a). Degenerative processes in the scala media complex are present and very similar to those of the *v*, *sh*-1, *sh*-2, *je*, *pi* and *Va* group, but there are no anomalies of the vestibular apparatus. Work in progress in this laboratory has shown that there are several genetically distinct types of uncomplicated inherited deafness in the mouse, and the number of such conditions may well prove large.

6. Defect of the Otoliths

With the exception of Twirler, in all the conditions described above the otoliths are present when a given macula is present. However, in the recessive gene for pallid (symbol *pa*) which dilutes fur and eye colour, one or several otoliths are frequently missing (Lyon, 1951, 1953, 1955), while the maculae as such are apparently perfectly normal. When all four otoliths are absent, the mouse fails to respond to a change in position. This response is restored by the presence of a single otolith in a sacculus, which evidently means that the sacculus has

an equilibratory function in mammals. When otoliths are present on one side only, the head, in most pallids, is tilted with the abnormal side turned uppermost; these postural anomalies tend to disappear as the animals grow older, though the absence of otoliths persists unchanged.

7. Conclusions

The importance to sense physiology and otology of the material reviewed in this paper is obvious. Several additional genes are available for study, and new ones keep on being discovered. Much remains to be done even in the mutants discussed above. For instance, the six mutants of the first group should be compared on an isogenic background. On the technical side, the type of fixation and the celloidin embedding essential for good preparations of the labyrinth have so far restricted the range of histological methods which can be applied. The most serious gap in our knowledge is the absence of any reliable information about the central nervous system of these mutants. The facts presented here have been obtained by a small group of mammalian geneticists with interests in embryology and pathology. Further progress will largely depend on the degree of co-operation with specialists in related fields.

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DR M. R. DIX received her medical and surgical training at the Royal Free Hospital School of Medicine, London, and on graduation in 1937 was appointed House Surgeon to the Ear, Nose and Throat Department at the Royal Free Hospital. She held subsequent appointments as House Surgeon and Casualty House Surgeon at the General Hospital, Birmingham, and obtained the F.R.C.S. in 1943. In 1945 she became a member of the Scientific Staff of the Medical Research Council, and took part in clinical work embodied in the Report of the Medical Research Council's Committee on Electro-Acoustics: *Hearing aids and audiometers* (*Spec. Rep. Ser. med. Res. Coun., Lond.* 1947, No. 261). Subsequent work with the Otological Research Unit at the National Hospital, Queen Square, has been concerned with the development of the "peep-show" technique for the diagnosis of deafness in young children; investigation of the toxic effects of streptomycin upon the eighth nerve system, and the development of various new clinical methods of neuro-otological diagnosis. She has published papers (jointly) in the *Proceedings of the Royal Society of Medicine*, *Brain* and the *Journal of Physiology*. She was awarded the Harrison Prize in Otology by the Royal Society of Medicine in 1954.

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MR JOHN EDWARD SEARS joined the National Physical Laboratory in 1910 and became the first Superintendent of the Metrology Division, a post he held from 1918 until he retired in 1946. In this capacity he was responsible for maintaining the laboratory standards of measurement in length, mass, time, area, volume, density and atmospheric pressure, as well as for the associated verification work, including the testing of precision engineering measuring equipment and gauges. During 1921–32 he was concurrently deputy warden of the standards at the Board of Trade. His services on committees of the British Standards Institution were always in demand, particularly as chairman, and he was a member of the Hodgson Committee of the Board of Trade which reported in 1951 its recommendations for the revision of weights and measures legislation. In 1930 he was elected the member for the United Kingdom on the International Committee of Weights and Measures and was President of this Committee from 1946 until shortly before his death on 21 December 1954, at the age of 71. Among the work with which his name is especially associated may be mentioned the determinations of the yard and the metre in wavelengths of light, and of the refractive index and dispersion of air.

DR I. C. WHITFIELD was educated at University College, London. After working for a time in the research laboratories of May & Baker Ltd. at Dagenham, he went to the University of Aberdeen as assistant lecturer in the Physiology Department, where he was engaged in research on problems in the circulatory field. He later turned his attention to neurophysiology and took up the post of Lecturer in Physiology at the University of Birmingham. There he has been engaged on teaching and research in neurophysiology, and has conducted a number of investigations of the electrophysiology of the cortical connexions of the cochlear nerve, including the cochlear nuclei and auditory cortex. Dr Whitfield—in addition to his publications upon the central auditory pathways—is the author of *An introduction to electronics for physiological workers* (1953), and of papers upon reaction kinetics, the circulation, electroencephalography and on electronic methods in physiology.

* * *

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Scientific Editor: W. D. M. Paton

Physiology of Voluntary Muscle: Introduction

Brains without muscle would not get animals very far, or the human body out of an arm-chair. The capacity to move is a dominant fact in biology. It is not surprising therefore that the study of muscles and of muscular motion has attracted many of the best workers in the field of physiology. It may be recalled that the Croonian lecture of the Royal Society was founded in 1701 with its main subject, " The advancement of natural knowledge on local motion ". Some of the broader conceptions in physiology, biochemistry, and biophysics have revolved round muscular tissue. Thus we had in the last century the original conception (Hermann-Pflüger) of an *inogen* molecule, thought to be built up with oxygen and other constituents to a complex, which on stimulation explosively re-arranged to give CO_2, myosin and lactic acid. As so often happens, the inogen theory had become inhibitory to progress; it remained for Walter M. Fletcher in Cambridge[1] to show that in surviving amphibian muscle the main evolution of CO_2 takes place after the contraction of the muscle is over, and when oxygen is admitted. The giant molecule conception was thus disproved, and in fundamental work with F. Gowland Hopkins these simple observations were much extended by the study of the relation of lactic acid appearance to contraction and oxygen usage. So the significance of studying simple chemical changes in tissue became clear, and the modern approach to this aspect of biochemistry, stabilized.

The entry of A. V. Hill into the field in 1910, with his studies of heat evolution, was crucial at this stage; the pure biocnemistry being later extended by Parnas, Meyerhof (a pupil of Otto Warburg), Embden and other schools. In this way muscle came to take its place with yeast as background material in the evolution of our knowledge of the more chemical aspects of physiology—with the difference that, in muscle, biochemists can never lose sight of the integration of the chemistry with the physiology. What a rich harvest has come! If we leave aside the more physiological aspects, we have the discoveries of " phosphagen ", of

[1] Later first Secretary of the Medical Research Council.

adenosine-triphosphate, the independent findings by Meyerhof and D. M. Needham of coupled oxidation and phosphorylation, with the subsequent studies of oxidative phosphorylation by Belitzer and Tsybikowa, foreshadowed by Kalckar for kidney, and independently on brain tissue by Ochoa in Oxford; finally came the conception of storage of energy in phosphate bonds (Lipmann), and the tricarboxylic acid cycle (Krebs) following on Szent-Györgyi. Now, after Lundsgaard's blow to the lactic acid theory in the early 1930's, and the discarding of other subsequent ideas, we notice a return to the central enzyme problem with the finding that myosin, itself a substantial part of the muscle substance, has enzymic properties, and to the study of changes in muscle tissue connected with plasticity. Most of the points mentioned, and several others, are to be found in the accompanying condensed reviews on muscle.

The volume, to which these remarks form an introduction, attempts to give an over-all picture in perspective of the present background of knowledge upon the physiology of voluntary muscle. It cannot claim to be complete, but if it whets the appetite for further study at some point it will have been useful; most of all, if it could bring conviction that practical results can follow with certainty only when the background of knowledge is complete. No one would think otherwise in engineering; why should they think differently in medicine?

This might have been written quite easily from the point of view of the so-called non-academic research. Those who live in the trenches (as it were) of medicine are apt to chafe at the Ivory Tower; as was once said to the writer, "Why cannot biochemists do something sensible like learning 'how to estimate the proteins of cerebrospinal fluid'!", a point of view which omitted to realize that the first study must be of proteins, so that we could at least know what it is that has to be estimated. It is regrettable but true that even when the pathological changes of disease are understood, the major advances required for therapy lie in an understanding of the normal physiology and biochemistry of the tissue concerned. As will be seen, for instance, from the article by B. McArdle, it has taken time to probe the abnormalities in the metabolism of potassium occurring in familial periodic paralysis. It would not be difficult to show that most of the academic research of the last 40 years (reviewed in this volume) is necessary to provide a basis for a complete understanding of this single condition alone.

As a general background, the article by A. V. Hill upon the design of muscles puts the whole subject in its place and gives an idea of the limitations which the land animal must face. In some respects his arguments are food for the philosophers. In the same wide biological strain we have James Gray and C. F. A. Pantin writing as zoologists upon muscular activity during locomotion, and upon the comparative physiology of muscle respectively. In this we come up against the extraordinary scope of adaptation in the animal kingdom; as Pantin puts it, in the fly the speed of muscular contraction in the wing muscles is less than 1 msec., whereas in the sea-anemone a twitch may take six minutes. These set limits for the underlying chemical changes. In the paper by R. G. Bannister on muscular effort, his conclusion that speed in sprinting is limited by the maximal contractile activity of the muscle, and that in long-distance running the rate of supply of oxygen and metabolites in muscle is all-important, focuses clearly the significance of energy supply, both anaerobic and aerobic, and all that this involves. His final remarks, as an athlete, on the psychological factors which override bodily limitations will be read with interest.

In a thoughtful paper, P. A. Merton deals with muscular fatigue, in which he shows how near to fatigue the muscles really live. How the regulation of the contraction of groups of muscle is related to the physiological needs and the willed requirement of the man (or animal) is reviewed in the article by P. H. Hammond, P. A. Merton and G. G. Sutton on nervous control of muscular contractions; so much has been contributed by Sherrington and his pupils (cf. the initial work on the "stretch reflex" by Liddell & Sherrington); among others the names of Eccles and of Granit are to be noticed, as also the latter's phrase describing the muscle spindles as the "private measuring instruments of the muscle's servo-mechanisms". The modern term "feedback" can also be appropriately used. B. Katz discusses the role of the cell membrane in muscular activity, emphasizing its dual duties of concern with excitation and with the maintenance of diffusion barriers. Its function is to pass on stimuli to set the muscle into activity, and the relation to acetylcholine enters at this point.

From these papers we pass to those giving a more detailed analysis. A. V. Hill summarizes the position of thermodynamics of muscle as he sees it after 46 years of work in a field where apparatus for heat measurement has been continuously improved. It should not be forgotten that, in the early 1920's, Hill shared a Nobel prize with Otto Meyerhof for their classical recognition that lactic acid is not completely burnt, but partly restored to the muscular apparatus

after contraction. Accompanying this is the summary of the present position of the mechanical properties of muscle by D. R. Wilkie. Those who remember the controversies upon the meaning of the bands of muscle striation will read with close attention the advances in this direction given by A. F. Huxley, writing on the evidence of visible light microscopy, and by H. E. Huxley upon the ultrastructure of striated muscle as seen by the electron microscope.

Thence we come to the problems of the intimate biochemistry of the muscle contraction. In a summary of the present knowledge of energy production in muscle, D. M. Needham, who has worked with distinction in this field for over 35 years, shows how each successive discovery has pushed back the substance to which we must look for contraction itself. Now it has come to the muscle proteins and ATP, a step in which Russian workers Engelhardt and Ljubimowa took part, and to which Szent-Györgyi's finding of the second protein component, actin, was fundamental. Kenneth Bailey writes on muscle proteins, and is followed by S. V. Perry on the interaction of actomyosin and ATP. Last to be mentioned, E. C. Bate-Smith and J. R. Bendall consider the changes in muscle after death; in so far as this is concerned with the eating qualities of meat, one would say "just an applied problem"; the same is true of the study of the relation of rigor to forensic medicine. Yet it is extraordinary to learn that the cause of it is not lactic acid, as we had thought, but that it is ATP in another guise. To quote from these authors, "stiffening [of muscle] cannot occur until the ATP level has fallen more or less drastically".

The time taken by scientists to reach results, and the apparent remoteness of their withdrawal from the obvious practical problem, must be exasperating to those who have to answer questions about their work and its finance; but even so, as has been often stressed before, this insistence upon close study of the basic problems seems a main way to get practical results, as well as illumination of knowledge. Will man ever try to imitate the muscle with his machines?

Rudolph Peters

We are indebted to Sir Rudolph Peters, Chairman of the planning committee of this symposium and author of the Introduction.

Notes on Contributors

DR KENNETH BAILEY has recently contributed to the Bulletin a paper, in conjunction with F. R. Bettelheim, on the nature of the fibrinogen-thrombin reaction (*Brit. med. Bull.* 1955, **11**, 50) with which a biographical note was published.

DR R. G. BANNISTER is House Physician, The Brompton Hospital, London. He was Junior Demonstrator in Physiology, University of Oxford, while Harmsworth Senior Scholar at Merton College, 1950–52; House Physician to Professor G. W. Pickering and the Medical Unit, St Mary's Hospital, London, 1954–55; House Surgeon, The Radcliffe Infirmary, Oxford, 1955; and House Physician Department of Medicine, Postgraduate Medical School, Hammersmith Hospital, London, 1956. In 1955 Dr Bannister was awarded the C.B.E., and the William Hyde Award for research relating to physical education and medicine. His published work includes: "Stress and sport" (*Practitioner*, 1954, **172**, 63); with D. J. C. Cunningham and C. G. Douglas, "The carbon dioxide stimulus to breathing in severe exercise" (*J. Physiol.* 1954, **125**, 90); and with D. J. C. Cunningham, "The effects on the respiration and performance during exercise of adding oxygen to the inspired air" (*J. Physiol.* 1954, **125**, 118).

DR E. C. BATE-SMITH joined the staff of the Low Temperature Research Station, Cambridge, in 1926 to work on post-mortem changes in muscle. In 1939 he became Head of the Animal Products Section and in 1946 succeeded Dr F. Kidd, F.R.S., as Superintendent of the Low Temperature Research Station, which post he still holds. His present work is administrative but he conducts some personal research on phenolic constituents of foods. In 1952 he edited (with Mr T. N. Morris) *Food science: a symposium on quality and preservation of foods*, which was published by the Cambridge University Press. Important original publications are: "The physiology and chemistry of rigor mortis, with special reference to the aging of beef" (*Advanc. Food Res.* 1948, **1**, 1); with J. R. Bendall, "Factors determining the time course of rigor mortis" (*J. Physiol.* 1949, **110**, 47); and "The flavonoid compounds in foods" (*Advanc. Food Res.* 1954, **5**, 262).

MR J. R. BENDALL has, since 1942, been on the staff of the Low Temperature Research Station, Cambridge, first as Scientific Officer, and now as Principal Scientific Officer. From 1940 to 1942 he worked in the School of Biochemistry, Cambridge, on biochemical aspects of shock. From 1942 until 1945 Mr. Bendall's work concerned the preparation of special rations for the Army, and since that time he has worked on the biochemistry of muscle in relation to the technology of meat. He has published papers on factors determining the time-course of rigor mortis (with E. C. Bate-Smith, *J. Physiol.* 1949, **110**, 47); on relaxation of muscle fibres (*J. Physiol.* 1953, **121**, 232); and on the relaxing effect of myokinase on muscle fibres (*Proc. roy. Soc.* B, 1954, **142**, 409).

PROFESSOR SIR JAMES GRAY has been a Fellow of King's College, Cambridge, since 1914, and Professor of Zoology, University of Cambridge, since 1937. He is Development Commissioner and Chairman of the Fishery Advisory Committee, late President of the Marine Biological Association, and Trustee of the British Museum. From 1942 to 1947 Sir James was a member of the Agricultural Research Council, and from 1943 to 1947 he was Fullerian Professor of Physiology, Royal Institution. He is the author of *Ciliary movement* (1928), *Textbook of experimental cytology* (1931), *How animals move* (1953); and of various scientific papers.

MR P. H. HAMMOND is a Senior Scientific Officer at the Radar Research Establishment, Malvern. He studied electrical engineering at the Sunderland Technical College and later at King's College, Newcastle-on-Tyne, and has since been engaged on research work in the servo-mechanism field. He has published papers on electronic simulators for hydraulic servo-mechanisms and on an infra-red monochromator with constant energy output.

PROFESSOR A. V. HILL is Research Associate in the Department of Physiology, University College, London. He was a Fellow of Trinity College, Cambridge (1910–16), Fellow of King's College, Cambridge (1916–22), Professor of Physiology, Manchester University (1920–23), Professor of Physiology, University College, London (1923–25), Research Professor of the Royal Society (1926–51), Secretary of the Royal Society (1935–45), and Member of Parliament for the University of Cambridge (1940–45). His work has been concerned with physiology, biophysics, physical chemistry, instruments, and air defence. Among his published papers are the following: "The energy degraded in the recovery processes of striated muscles" (*J. Physiol.* 1913, **46**, 28); "The heat production of nerve" (*J. Pharmacol.* 1926, **29**, 161); "The heat of shortening and the dynamic constants of muscle" (*Proc. roy. Soc.* B, 1938, **126**, 136). Professor Hill was elected Fellow of the Royal Society in 1918, and awarded the Nobel Prize for Physiology and Medicine in 1922. He was made Companion of Honour in 1946, and awarded the Medal of Freedom (USA) in 1947 and the Copley Medal of the Royal Society in 1948.

NOTES ON CONTRIBUTORS

MR A. F. HUXLEY is a Fellow and Lecturer of Trinity College, Cambridge, and an Assistant Director of Research in the Physiological Laboratory of the University. He spent the war years in operational research in gunnery, first for Anti-Aircraft Command and later for the Navy. On returning to Cambridge in 1946, he joined Professor A. L. Hodgkin and they took up work on the giant axon of the squid that had been begun by them in 1939 but was interrupted by the outbreak of war. This led to an explanation of the nerve action potential in terms of changes in the permeability of the surface membrane to sodium and potassium ions. With Dr R. Stämpfli, he investigated conduction in myelinated nerve fibres. Since 1951, his interests have turned towards muscle. He developed an interference microscope for studying isolated muscle fibres, and has investigated the role of the striations in muscle by means of this instrument and by other optical and electrical methods. He was elected a Fellow of the Royal Society in 1955. He was Press Editor of the *Journal of Physiology* from 1950 until early this year, and is now Chairman of its Editorial Board.

DR H. E. HUXLEY read Natural Science Tripos Pt I at Cambridge, 1941–43, and worked on radar in the Royal Air Force, 1943–47. He read Pt II Natural Science Tripos (Physics), 1947–48. He was Research Student in the Medical Research Council (MRC) Unit, Cavendish Laboratory, Cambridge, 1948–52, when he gained his Ph.D. Dr Huxley held a Commonwealth Fund Fellowship for study and travel in the USA, 1952–54, where he worked under Professor F. O. Schmitt in the Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. He returned to Cambridge in 1954 to work again in the MRC Unit, and was also a Fellow of Christ's College. He moved to the Department of Biophysics, University College, London, in 1956, as Research Associate, and member of the External Staff of the MRC. Dr Huxley worked on *x*-ray diffraction by crystalline proteins, then made similar studies on muscle, taking up electron microscopy in the USA, and later phase-contrast and interference microscopy. His main interests are electron microscopy, molecular structure, and muscle. His papers on studies on muscle have been published in *Proc. roy. Soc.* B, 1953, **141**, 59; *Biochim. Biophys. Acta*, 1953, **12**, 387; *Symp. Soc. exp. Biol.* 1955, **9**, 228 (with J. Hanson).

PROFESSOR B. KATZ has been Professor and Head of the Biophysics Department, University College, London, since 1952. From 1946 to 1950 he was Assistant Director of Research, Biophysics Research Unit, University College, and Henry Head Research Fellow (Royal Society). He was Reader in Physiology, 1950–51. In 1952, Professor Katz was elected a Fellow of the Royal Society. His work has been concerned for some years with the physico-chemical mechanisms of neuromuscular transmission, and a series of papers on this subject, in conjunction with Dr P. Fatt and Dr J. del Castillo, have appeared in the *Journal of Physiology*. The principal method used in this study was the intracellular recording of the membrane potential at the end-plates of single muscle fibres. Professor Katz is also the author of *Electric excitation of nerve* (1939).

DR BRIAN MCARDLE is on the Scientific Staff of the Medical Research Council, and has been working at Guy's Hospital, London, since 1946, for the most part in the Clinical Research Unit, but since 1955 in the Department of Chemical Pathology. After holding various resident posts at Guy's Hospital, the Hospital for Sick Children, Great Ormond Street, London, and the Brompton Hospital, London, he went for three years to the Department of Medicine in the University of Cambridge as Elmore Medical Research Student. During the war his work, largely at the National Hospital for Nervous Diseases, Queen Square, London, was concerned with motion sickness and the effects of heat. His abiding interest has been in neuromuscular disorders. Among his publications are: "Myopathy due to a defect in muscle glycogen breakdown" (*Clin. Sci.* 1951, **10**, 13), and, jointly, "Blood pyruvate estimations in the diagnosis and treatment of polyneuritis" (*Brain*, 1950, **73**, 431) and "Responses to ischaemic work in the human forearm" (*Clin. Sci.* 1956, **15**, 305).

DR P. A. MERTON read physiology at Cambridge as a pupil of Dr W. A. H. Rushton and for a short time did biochemical research under Professor D. Keilin. Since 1946 he has worked for the Medical Research Council in Dr E. A. Carmichael's unit at the National Hospital, Queen Square, except for two seasons with Ragnar Granit in Stockholm. He started to work on diseases of muscle, and later turned his interest to problems of muscular co-ordination and performance; subsequent papers (in the *Journal of Physiology*) being mainly an expansion of this work.

DR D. M. NEEDHAM works at the Biochemical Laboratory, Cambridge. Under Professor Sir F. G. Hopkins she began in 1920 with problems of muscle contraction, and the still unsolved puzzles of the intimate mechanisms involved have remained her chief interest. She has published many papers and reviews on this subject and on carbohydrate metabolism. Her book, *Biochemistry of muscle*, was published in 1932. She has held a Beit Memorial Fellowship and grants from the Medical Research Council. At present she is working for the Agricultural Research Council on the biochemistry of the uterus, especially the contractile mechanism. She was elected a Fellow of the Royal Society in 1948. During the war she joined her husband (in charge of the Sino-British Science Co-operation Office) in Chungking. In the endeavour to bring aid to Chinese scientists and technologists carrying on under the conditions of the blockade, they travelled by truck all over unoccupied China. The deep impressions of that time are recorded in their book, *Science outpost* (1948).

DR C. F. A. PANTIN has been since 1937 Reader in Invertebrate Zoology, University of Cambridge. He was Physiologist to the Marine Biological Laboratory, Plymouth, 1922–29, and Lecturer in Zoology, University of Cambridge, 1929–37. His work has been chiefly concerned with the elementary nervous system, the relation of animals to environment, particularly osmotic regulation, physiological influences on animal distribution, muscular action in invertebrate animals and comparative functional histology. His work covers most of these fields, though his main line continues to be the study of the elementary nervous system. He delivered the Royal Society Croonian Lecture on this subject in 1952 (*Proc. roy. Soc.* B, **140**, 147), and an address to the British Association in 1951, on organic design (*Advanc. Sci.* **8**, No. 30). In conjunction with E. J. Batham, he wrote a paper entitled "The organization of the muscular system of *Metridium senile*" (*Quart. J. micr. Sci.* 1951, **92**, 27). Dr Pantin was, in 1937, elected a Fellow of the Royal Society, and given the Trail Award of the Linnean Society of London; in 1950 he was awarded the Royal Medal of the Royal Society. He holds an Honorary Doctorate of the University of Sao Paulo, and is an Honorary Member of the Royal Society of New Zealand.

PROFESSOR W. D. M. PATON, who has acted as Scientific Editor for this number of *British Medical Bulletin*, is Professor of Pharmacology, Royal College of Surgeons of England. He previously contributed to the Bulletin an article on ganglionic blocking agents (*Brit. med. Bull.* 1952, **8**, 310), with which a biographical note was published. Professor Paton is Secretary of the Physiological Society, and in 1956 he was elected a Fellow of the Royal Society.

DR S. V. PERRY was, from 1947 to 1951, Research Fellow, Trinity College, Cambridge. From 1948 to 1949 he held a Commonwealth Fund Fellowship in the Department of Physiology, University of Rochester, Rochester, N.Y., and in 1950 he was appointed Lecturer in Biochemistry, University of Cambridge, which post he still holds. His earlier work was concerned particularly with the isolated muscle proteins, but for the last seven years his interest has been focused on the intracellular organization of the muscle cell. This work has involved morphological and biochemical study of myofibrils and mitochondria isolated from skeletal muscle tissue. His current work is concerned with nucleotide metabolism in the muscle cell, particularly in relation to relaxation, and the localization of the protein components of the myofibril. Among many papers published in scientific journals are the following: with K. Bailey, "The role of sulphydryl groups in the interaction of myosin and actin" (*Biochim. Biophys. Acta*, 1946, **1**, 506); "The adenosinetriphosphatase activity of myofibrils isolated from skeletal muscle" (*Biochem. J.* 1951, **48**, 257); "Relation between chemical and contractile function and structure of the skeletal muscle cell" (*Physiol. Rev.* 1956, **36**, 1).

SIR RUDOLPH PETERS is now a member of the Scientific Staff of the Agricultural Research Council, Institute of Animal Physiology, Cambridge, and is Emeritus Professor of Biochemistry, University of Oxford. He has written several papers for the Bulletin—among them, "Significance of biochemical lesions in the pyruvate oxidase system" (*Brit. med. Bull.* 1953, **9**, 116). Biographical notes have been published in *Brit. med. Bull.* 1945, **3**, 69; and *Brit. med. Bull.* 1956, **12**, 88).

MR G. G. SUTTON is Principal Scientific Officer at the Radar Research Establishment, Malvern. He has been in the Scientific Civil Service since the beginning of the last war. He qualified in engineering at Queen Mary College, University of London. His previous published work includes a study of skin effect in materials at high frequencies, the design of an automatic spectrophotometer and the design and use of equipment for the study of a subject working in closed loop control systems.

DR D. R. WILKIE was appointed House Physician, Medical Unit, University College Hospital, in 1945; and Lecturer, Physiology Department, University College, London, 1946–48 and 1951. He held the rank of Flight Lieutenant at the R.A.F. Institute of Aviation Medicine, Farnborough, from 1948 to 1950. He was Locke Fellow of the Royal Society from 1952 to 1954, and in 1954 became Reader in Experimental Physiology at University College, London. Since leaving clinical medicine, Dr Wilkie has worked on muscle physiology, except for a period in the RAF when he worked on acoustic problems, especially the design of noise-excluding helmets. Among his publications are: "The relation between force and velocity in human muscle" (*J. Physiol.* 1949, **110**, 249); "The acoustic properties of flying helmets" (*J. acoust. Soc. Amer.* 1952, **24**, 191); "Facts and theories about muscle" (*Progr. Biophys.* 1954, **4**, 288).

THE DESIGN OF MUSCLES

A. V. HILL F.R.S.

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University College, London

1 Biological " design "
2 Body-size
3 The speed of muscles
4 Limiting factors—body-temperature: diffusion: structural strength
References

1. Biological " Design "

In astronomy, as in chemistry and physics apart from their engineering applications, the ideas of function, adaptation, purpose or survival have no place: but these are the basic concepts of biology. They are not the result of preconception or philosophy, but simply of direct observation of how living things behave. Their recognition does not demand acceptance of any particular theory of how the essential peculiarities of life emerged, if indeed they did emerge, from the properties of non-living matter. The practical biologist need not take sides in disputes about vitalism, mechanism and teleology. In the physical world it is clearly meaningless to ask about the function of carbon dioxide, or the purpose of the solar system or the solid state: but in biology it is just as sensible to inquire about the function of an organ, or the purpose of an adaptation or reaction, as it is in engineering about the object of a structure or mechanism. The word " design " need mean no more, and no less, to a biologist than it does to an engineer. Its use in the title of this article implies simply a recognition of the plain fact that, over a very wide range of conditions, muscles (like other organs) are well adjusted to serve the purposes for which they are employed. Essential connexions indeed will be missed by regarding muscles simply as problems in chemistry and physics: to the experimental methods of the chemist and physicist must be added the functional approach of the biologist or engineer.

In an introductory chapter, on general physiology, to his Silliman Lectures on blood, Lawrence J. Henderson (1928) wrote:

> The law of adaptation in organisms, founded upon the fact of survival, seems to be quite as well established as the second law of thermodynamics, and almost equally serviceable.

The adaptation referred to by Henderson is that of an animal or organism, either to a constraint imposed on it from outside, or to the internal effects of injury or disease. But it applies equally to the appropriateness of the design, construction and reactions of the body, as a whole or in detail, to its normal needs and environment. To come specifically to muscle, we can be as sure as we should be about a deduction from the second law of thermodynamics that every muscle, or group of muscles, will show, qualitatively and quantitatively, the sort of properties that a very intelligent engineer, knowing all the facts, would have designed for them in order to meet, within wide limits, the requirements of their owner. These properties, of course, must provide a compromise between the various needs, and it is never safe, because one sees a particular need, to assume that a muscle is ideally designed to meet that one alone: under other conditions other demands also must be met. For example, the possibility of speed of movement is often a very desirable quality, but so is economy of energy in maintaining force or posture: and faster muscles are more wasteful in postural effort, so a balance must be struck between the two requirements. The intrinsic speed of a muscle is inherent in the muscle itself; it depends on physico-chemical factors in its structure and mechanism, not mainly on control by the nervous system—though that also is adjusted to the animal's requirements. Again, for a given weight of muscle, a greater force can be developed if more and shorter fibres are arranged approximately in parallel: but this limits the range of possible shortening, and an anatomical compromise is necessary according to the position and function in the body. In most animals the energy for muscular effort is obtained largely by burning carbohydrate: but hydrated glycogen is a very heavy fuel compared with fat and, in the locust which flies great distances (over water or desert), a sufficient load of glycogen would severely limit range and endurance. The animal therefore rapidly transforms the carbohydrate of its diet into fat, which alone is used during flight (Weis-Fogh, 1952).

2. Body-Size

Oxygen supply is a major factor in endurance and depends in vertebrates on the output of the heart; and the more athletic animals have rather larger hearts. But larger hearts would be no good without larger lungs to oxygenate the blood and larger vessels and more capillaries to allow it to pass: there is clearly a limit to the volume of the circulatory system in an " all-purpose " animal. The basal metabolic rate is approximately proportional to the body surface: in cold surroundings this is a condition of maintaining body-temperature. But in warm surroundings the upper limit of continued exertion is set, as Marathon runners find, by the need to get rid of waste heat; so the maximum power output in maintained effort also is proportional to body surface.[1] This fits in with the fact that the larger animal must, for dynamical reasons, have muscles of intrinsically lower speed, i.e. they take longer to shorten a given fraction of their length and carry out fewer cycles of movement a minute (Hill, 1950). Moreover the heart rate is less in bigger animals, varying inversely about as the linear size; this also is necessary for purely dynamical reasons, one could not expect a heart weighing half a ton (in the blue whale) to beat as fast as one weighing a few centigrams (in the humming-bird). But the physical mechanism by which the speed of cardiac cells is adjusted to body-size is no more obvious than was, before Newton, the answer to the question of why an apple falls to the ground.

3. The Speed of Muscles

This general reasoning allows us, not to make positive prediction, but to put questions for experimented answer. Every muscle has two optimum speeds: one for maximum efficiency (work done/energy used), one for maximum power output (work done per minute). In a well-designed engine of

[1] See article on " Muscular effort " by R. G. Bannister, p. 222 of this number of the Bulletin, for discussion of the limiting factors in athletics.—ED.

human construction these two speeds are about the same—which is found to be true also in muscles. At extremes of speed, very high and very low, efficiency and power output become small. The optimum speed of a muscle is 20–30% of the maximum speed at which it can shorten under zero load (its "intrinsic speed"). Now the intrinsic speed of muscles varies enormously from one muscle to another in the same animal, and between similar muscles of animals of different size. The oculo-motor muscles are very rapid compared with skeletal muscles, particularly those that are chiefly concerned with maintaining posture. The jumping muscles of the kangaroo are intrinsically very slow compared with those of the desert rat, although their anatomical design is similar, and both animals can jump about the same distance. Heart rates in mammals must vary from the smallest to the largest in a ratio of a hundred or more to one; and all of them we should expect to work at a speed not too far from their optimum. Now this provides a question of great physiological interest, the sort of question that this kind of reasoning prompts us to ask. The heart rate in a human athlete may be about 45/min. at rest, about 70/min. during moderate exercise, about 180/min. during maximum effort. If the heart were an ordinary skeletal muscle, and if its efficiency (work done/energy used) were near the maximum under conditions of severe effort, it would be rather low under conditions of rest or moderate exercise. If its efficiency were near its maximum with moderate exercise, it would be low with maximum effort. Perhaps this is so, and a compromise has had to be made. But the possibility remains that cardiac muscle may be able to change its intrinsic speed according to conditions: the question suggested is whether the heart, unlike the skeletal muscle, becomes intrinsically quicker when the frequency of its beat rises. It should not be too difficult experimentally to answer this question, but there is no evidence at present as to what the answer is.

4. Limiting Factors—Body-Temperature: Diffusion: Structural Strength

There are certain physical factors, the effects of which millions of years of evolution have not, except indirectly, been able to avoid. One of these is the influence of temperature: the speed of everything that can be measured in muscle is diminished two or three times by a fall of temperature of 10° C. This provides the severest limitations in the behaviour of land animals liable to encounter considerable changes of temperature, and it led to the evolution of a mechanism for maintaining constant body-temperature. The speed of diffusion of dissolved substances in solution is another limiting factor: in objects of similar shape the time taken for diffusion to operate varies directly as the square of the linear size. It is necessary, therefore, particularly for an adequate supply of oxygen, that the middle of a cell should not be too far from the nearest blood capillary. It might have been imagined that, in similar animals of different size (e.g. the dolphin and the whale), the bigger animal would have muscle cells of greater diameter in proportion to its length. But consider a whale ten times as long as a dolphin. If its muscle cells were of ten times the diameter, their maximum rate of oxygen consumption (per gram) would be reduced to one-hundredth. Obviously they could not work under such conditions. The total power output of similar animals increases about as the square of their linear size, so the maximum oxygen requirement per gram diminishes inversely as the linear size. The diameter of the muscle fibre of the whale, therefore, might be $\sqrt{10}$ times—but it certainly could not be 10 times—that of the dolphin. A comparison of fibre diameters, in similar muscles of similar animals of different size (dolphin and whale, shark and dogfish, greyhound and whippet, cat and tiger, etc.), would be of considerable interest.

Another limiting factor is the absolute strength of the structural material with and on which skeletal muscles operate. Such accidents in athletics as the tearing of tendons and muscles show that the factor of safety is not very great. At the expense of less economy and greater fatigue in maintaining load or posture, it would have been possible to supply an animal with quicker muscles: but it seems likely that, if this had gone far, the greater accelerations so available would have provided stresses that the structures could not bear, and frequent damage would result. An animal's muscles could be quickened about 20% by raising its body-temperature 2° C. This could be rapidly achieved by powerful diathermy, and—if it did not upset nervous control—it would be interesting to see if a good runner could then do 100 yards in 8 seconds. The experiment, however, would be dangerous for, were it successful, serious mechanical damage might occur: it would be better to try it first on a whippet!

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INTERPRETATION OF MUSCLE STRIATION: EVIDENCE FROM VISIBLE LIGHT MICROSCOPY

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1 Observations during the 19th century
2 Measurements on surviving muscle fibres with the interference microscope
3 Measurements on separated myofibrils with the phase-contrast microscope
4 Interpretation of the A, I and H bands
5 Activation and the Z line
References

Our understanding of the structure of striated muscle has advanced greatly during the last few years. This is the result partly of the application of new physical methods such as small-angle *x*-ray diffraction and electron microscopy, partly of new knowledge of the structural proteins of muscle, and partly of the revival of light microscopy, especially the development of phase-contrast and interference methods which give much more faithful images of fresh, unstained material than can be obtained with ordinary, or "direct" (Baker, 1956), microscopy. These diverse methods have given results which fit together very satisfactorily, and it is now possible for the first time to give interpretations for all the main features of the striations. This article will describe the evidence that has been obtained by microscopy with visible light; the other lines of evidence are discussed in the articles by Bailey[1] and by H. E. Huxley[2] in this issue of the Bulletin.

[1] p. 183.—Ed. [2] p. 171.—Ed.

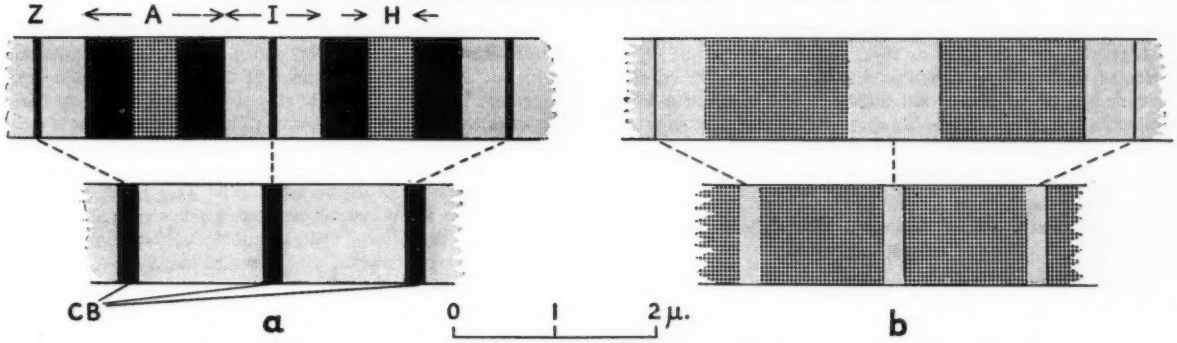
1. Observations during the 19th Century

The basic facts about the striations in a resting muscle fibre were discovered about a hundred years ago. The foundations were laid by the famous paper of Sir William Bowman (1840), which finally disposed of the idea, previously current, that the striated appearance was due to ring-like structures at the surface of the fibre. He showed that each fibre consists of a mass of fibrils, about 1–2 μ. thick, lying parallel to the long axis of the fibre, the whole being enclosed in an apparently structureless membrane, the sarcolemma. The striation pattern is visible in each of these "myofibrils" if they are separated, and they are normally aligned so that corresponding points of the striation pattern are at the same level, giving the appearance of disks traversing the whole thickness of the fibre. Bowman suggested that the striations are visible because different parts of the system of bands have different refractive indexes, not because of differences in the absorption of light; this view has been amply confirmed both by direct microscopy and by phase-contrast and interference methods; it was universally accepted during the last century but appears often to have been forgotten in more recent years.

W. M. Dobie, of Edinburgh, described in 1849 a narrow line of high refractive index material (now known as the Z line) bisecting the less dense of the principal bands seen by Bowman. Brücke (1858) showed that the broad band of high refractive index is birefringent (that is to say, the refractive index of this band is very slightly higher if the electric oscillation of the light by which it is measured is parallel to the fibre axis than if it is perpendicular). This band is therefore optically anisotropic and is called the A band; the lower refractive index region is optically isotropic and is called the I band. The only other important feature of the striations is that the central region of the A band is slightly less refractile than the edges; this central region is known as the H band, after Hensen, who gave a rather unsatisfactory account of it in 1869; it was first well described by Englemann in 1873.

Between 1870 and 1900, much effort and much acute observation were devoted to the study of the changes which take place when the fibre contracts. It was established that "contraction bands" (fig. 1) are formed in a powerful contraction, while most investigators found that moderate changes

FIG. 1. DIAGRAMS SHOWING (a) DISTRIBUTION OF REFRACTILE MATERIAL AND (b) DISTRIBUTION OF BIREFRINGENT MATERIAL, AS ESTABLISHED IN THE 19th CENTURY



In (a) the refractile material is dark; in (b) the birefringent material is dark, as seen under the polarizing microscope with compensation.

Top: fibril at extended length in body
Bottom: strongly contracted fibril showing contraction bands (CB) when viewed by direct microscopy

of length took place mostly in the I bands. No satisfactory theory emerged to unify these observations, and, while the formation of contraction bands was confirmed during the present century on fixed fibres (Jordan, 1933), studies with photographic methods on surviving isolated fibres seemed to show that it was the A bands whose width changed most when a fibre contracted by a moderate amount, or was passively stretched (Hürthle, 1909; Buchthal, Knappeis & Lindhard, 1936). This was very difficult to reconcile with the formation of contraction bands at higher degrees of shortening; the question thus clearly needed re-investigation, and was attacked by different methods at the Physiological Laboratory of Cambridge University and at the Medical Research Council's Biophysics Research Unit, King's College, London; the latter work was continued at the Massachusetts Institute of Technology.

2. Measurements on Surviving Muscle Fibres with the Interference Microscope

The difficulties of observations of this kind are chiefly due to the fact that an unstained muscle fibre is a "phase object", i.e., its structure is visible because of differences of refractive index, not of light absorption. Direct microscopy is unsatisfactory for phase objects; it is astonishing that the 19th century microscopists were as successful as they were. They chiefly used muscles of insects in which the striations are broader than in vertebrates; fibres from the frog, on the other hand, have the great advantage that they respond normally to stimulation when isolated, while insect fibres do not. To overcome the greater optical difficulties of the frog fibre, a two-beam interference microscope was developed at Cambridge with the co-operation of Messrs R. & J. Beck (A. F. Huxley, 1952, 1954; phase contrast is not effective on a specimen as thick as this). The objective of this instrument has a numerical aperture of 0.9, and it was used with a condenser aperture of 0.5–0.6. A very satisfactory image of the striations in a whole fibre was obtained; by altering the setting of the compensator on the instrument, either the A or the I bands could be made to appear dark (Plate I, fig. A). It was immediately found (A. F. Huxley & Niedergerke, 1954) that when the fibre was stretched, or released and allowed to shorten passively, the A bands stayed of constant width (Plate I, fig. B). The same was true when the fibre was stimulated to contract, so long as the amount of shortening was not too great; slow contractions were recorded by cinematography, and twitches or short tetanic contractions by a series of flashes from a xenon discharge tube. When the fibre shortened so much that adjacent A bands came into contact, contraction bands began to be formed. No change in the widths of the bands was observed in twitches in which the fibre was not allowed to shorten.

3. Measurements on Separated Myofibrils with the Phase-Contrast Microscope

The optical problem was solved differently by Dr J. Hanson at King's College, London. She used the phase-contrast microscope, and observed not whole fibres but separate myofibrils obtained by breaking up muscle in a suitable medium. This procedure gives an excellent image of the striations. The fibrils cannot be made to shorten by electrical stimulation, since the membrane of the fibre has been destroyed, but contractions can be obtained by irrigating the fibrils with a solution containing adenosinetriphosphate (ATP) while they are under the microscope. In this way, the formation of contraction bands was very clearly demonstrated (Hanson, 1952). Later, in collaboration with H. E. Huxley, fibrils were studied at different degrees of stretch and during shortening induced by treatment with ATP. As in the interference microscope studies on whole fibres, the A band was found to keep a constant width (H. E. Huxley & Hanson, 1954). It was also found that, when a fibril is stretched, the H band increases in width by the same amount as does the separation between adjacent Z lines.

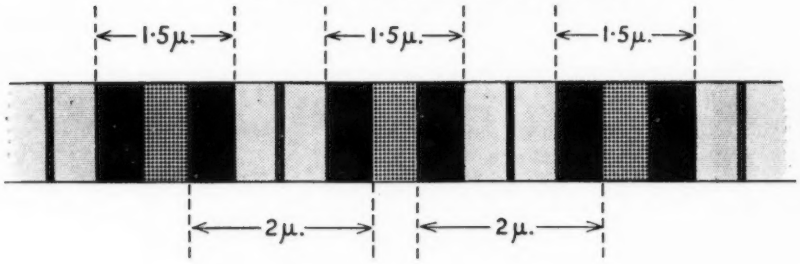
4. Interpretation of the A, I and H Bands

Thus, the two distances indicated by arrows in fig. 2 remain constant as the fibre is stretched; it is therefore natural to suppose that in these positions the muscle contains rodlets, or filaments, which do not change in length, but slide past one another as the fibre, or fibril, is stretched or shortened. The *x*-ray and electron-microscope observations of H. E. Huxley, described in his article elsewhere in this issue of the *British Medical Bulletin* (p. 171), also lead to this idea, which can therefore be regarded as very well supported, if not yet established beyond doubt. It is also strongly supported by extraction experiments in which the components are removed in turn by treatment with appropriate solutions; these experiments are also described by H. E. Huxley. A group of fibrils from which the material of the A-band filaments has been dissolved away is shown in Plate I, fig. C; it is seen that an almost empty gap appears in the position of the H band, agreeing with the interpretation of the nature of this band that was given above.

5. Activation and the Z Line

In the resting state, there is an electrical potential difference across the surface membrane of every muscle fibre, the interior being about 90 mv negative to the external fluid. If this potential difference is reduced by a sufficient amount, or actually reversed, the myofibrils inside the fibre contract. In the normal twitch of a mammalian muscle fibre, the change of membrane potential that sets off the contraction is the action potential itself; this is a short-lived ($\simeq$ 0.001 sec.) reversal of the membrane potential which propagates itself along the fibre by a mechanism closely similar to the conduction of a nerve impulse. During the action potential, considerable currents flow along the interior of the fibre, and it has been suggested that the fibrils are stimulated to contract by these currents flowing through them. This has been shown definitely not to be the case (Sten-Knudsen, 1954), and it is fairly clear that the essential thing is the change in the potential difference across the membrane itself. This poses the question, how does a potential gradient which exists only in the surface membrane itself bring about the contraction of fibrils in the interior of the fibre, which may be many microns away? A. V. Hill (1949) has shown that this cannot be explained by supposing that a substance is liberated at the membrane when the potential change occurs, and diffuses inwards to the fibrils; this would not be quick enough to account for the very rapid onset of a twitch. There must be some other specialized mechanism by which a reduction of membrane potential rapidly affects the fibrils within.

FIG. 2. DIAGRAM SHOWING THE DIMENSIONS THAT REMAIN CONSTANT AS A FIBRE IS STRETCHED



It has for some years seemed an attractive possibility that this inward conduction might take place along the Z membranes. The dense Z lines which bisect the I bands of each fibril appear to be connected together across the fibre to form more or less continuous membranes, which are attached to the sarcolemma at the surface of the fibre; these connexions are much more tenuous than the Z lines within the fibrils themselves, but they do seem to exist (Heidenhain, 1911; von Boga, 1937; Bennett & Porter, 1953). Evidence for this possibility has now been obtained (A. F. Huxley & Taylor, 1955a, 1955b). The method was to reduce the membrane potential of a very small area of the surface of a muscle fibre by applying a micropipette with a squared-off tip of diameter about 2 μ. to the surface of an isolated muscle fibre under a polarizing microscope. When a negative electric pulse was fed into this pipette, the potential difference across the area of membrane actually under the tip was reduced, while the rest of the membrane was practically unaffected. It was found that, when this was done with the pipette opposite a Z line (i.e., in the middle of an I band), that I band contracted in response to each electric pulse, but when the pipette was applied opposite an A band, so that its tip lay entirely in the interval between adjacent Z lines, no contraction occurred. The type of contraction is shown in Plate I, fig. D; a larger tip (4 μ.) was used in this case, to obtain a larger effect. The contraction spreads a considerable way towards the centre of the fibre, but the neighbouring striations are completely unaffected. These observations are strong evidence that "activation" is conducted inwards along some structure in the middle of the I band; it is very probable that this is the Z membrane.

A provisional interpretation is thus given for all the main features of the striation pattern. The A, I and H bands are due to the arrangement of two sets of filaments which slide past each other when the muscle shortens or lengthens, and the Z line activates the fibril in response to a reduction of the membrane potential. The light microscope has played a leading part in these developments.

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PLATE I. LIGHT MICROSCOPY OF MUSCLE

FIG. A. Photomicrographs of a small area of an isolated muscle fibre of the frog under an interference microscope. The fibre is immersed in a Ringer's solution containing serum albumin, to bring its refractive index to the mean value for the fibre. Repeat distance of striation pattern, 2.68 μ.

a: "positive contrast": high refractive index regions, including the A bands, appear dark.

b: "negative contrast": high refractive index regions appear light. There is a nucleus in the field of view; its refractive index is about equal to that of the I bands, while the nucleolus is denser even than the A bands.

Bottom strip: scale of distance, 10-μ. divisions

FIG. B. Passive stretch of an isolated muscle fibre of the frog. Interference microscope, negative contrast (A bands bright, I bands dark).

Note that the width of the A bands does not change appreciably as the fibre is stretched. The figure to the left of each photograph is the repeat distance of the striation pattern, in microns. The same nucleus is in the field of view in each picture.

Bottom strip: scale of distance, 10-μ. divisions

FIG. C. Removal of the A-band material from a group of myofibrils. Interference microscope, positive contrast (high refractive index dark). Fibrils prepared from glycerol-extracted rabbit muscle.

a: freshly prepared, in 0.1 M-KCl; A, I, Z and H bands are clearly seen.

b: same field after running 2 M-KI solution through; the dark lines are the Z lines, the A bands have disappeared altogether, and a narrow clear line is visible at the position of H. The Z lines are more conspicuous than in (a) because the compensator in the microscope has been re-set to give greater contrast.

c: another part of the same bundle of fibrils, where the gap at the position of the H band is more conspicuous.

Bottom strip: scale of distance, 10-μ. divisions

FIG. D. Shortening in a single I band produced by reducing the potential difference across a small area of the surface membrane.

a: before, and b–c: during, a negative pulse applied to the micropipette. Isolated fibre from the frog; polarized light, compensated so that A bands appear dark. Consecutive frames from a cine film.

Bottom strip: scale of distance, 10-μ. divisions

LIGHT MICROSCOPY OF MUSCLE

(FIGS. A—D)

A. F. Huxley

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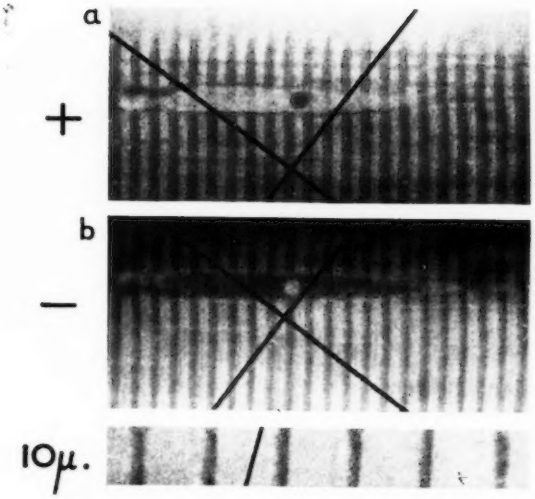

FIG. A

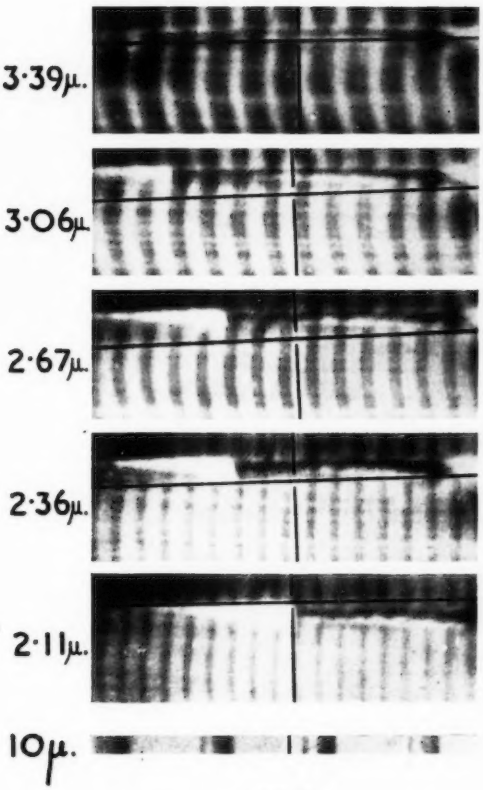

FIG. B

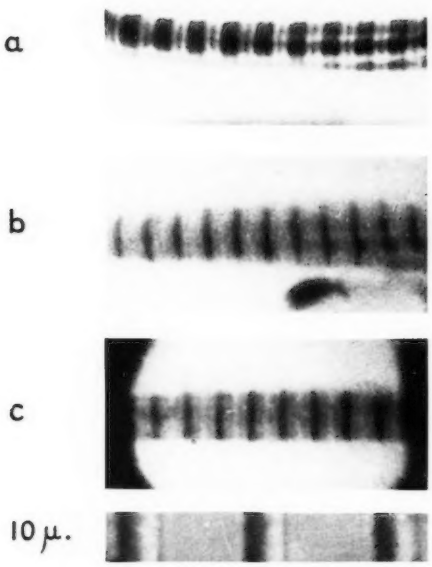

FIG. C

(Photomicrographs shown in figs. A and B were taken by the author in collaboration with Dr R. Niedergerke and those in fig. D by the author and Dr R. E. Taylor. Figure D is reproduced by permission of the Journal of Physiology.)

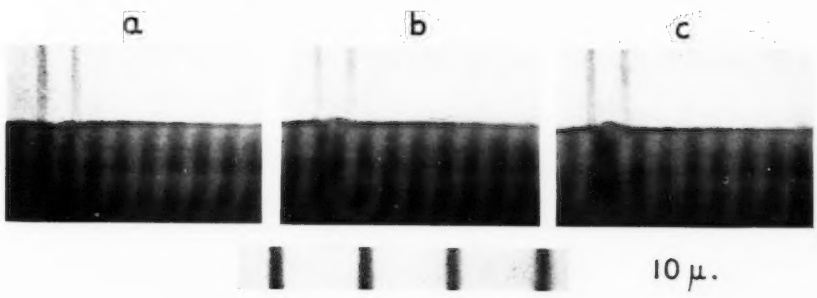

FIG. D

ULTRA-STRUCTURE OF STRIATED MUSCLE

(FIGS. A—C)

H. E. Huxley

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FIG. A (a)

FIG. B (a)

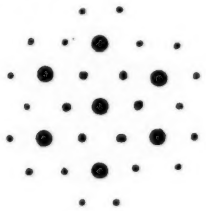


FIG. A. **Electron micrographs of striated muscle (rabbit psoas)**

a: cross-section through A band, showing double array of filaments

b: longitudinal section showing Z lines, the dense A band with well-marked array of filaments, and the less dense H zone in the centre of the A band

In the I bands the array of thick filaments is not seen

(magnification × 100,000)

FIG. A (b)

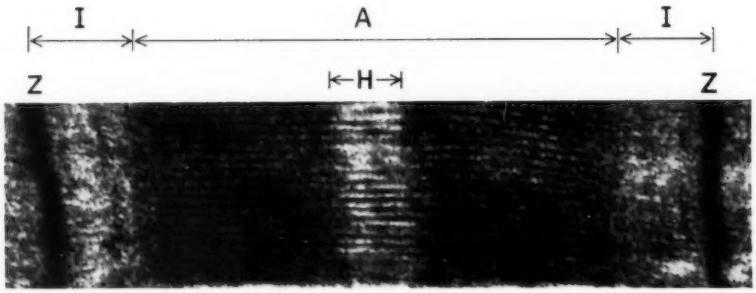


FIG. B (b)

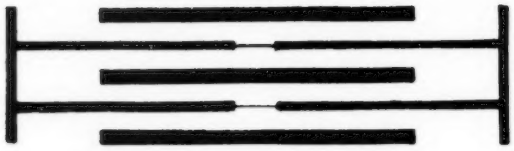
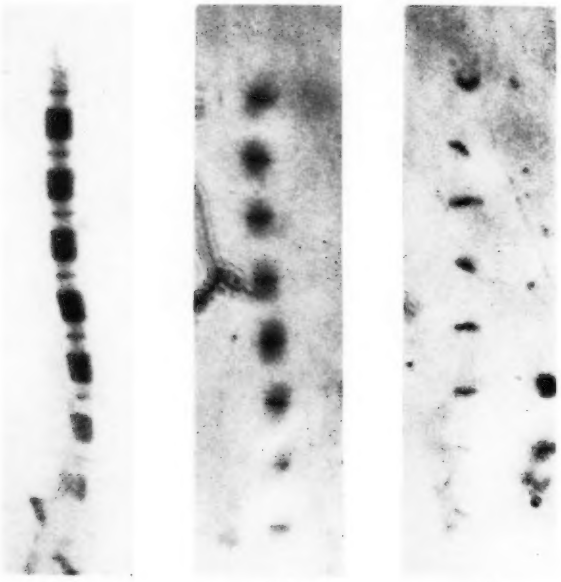


FIG. B. **Schematic diagrams of arrangement of filaments in striated muscle**

a: cross-section

b: longitudinal section

FIG. C



(a) (b) (c)

FIG. C. **Isolated myofibrils from rabbit psoas muscle, viewed in phase-contrast light microscope**

a: normal fibril (slightly stretched)

b: same fibril after extraction of myosin

c: same fibril after subsequent actin extraction

(magnification × 4,000)

THE ULTRA-STRUCTURE OF STRIATED MUSCLE

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1 Electron-microscope observations
2 The location of actin and myosin
3 Interference microscope observations
4 A model structure for muscle
5 Low-angle *x*-ray studies on muscle
6 Axial periodicities in the filaments
7 High-angle *x*-ray studies on muscle
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Muscle is a machine for converting chemically-stored energy into mechanical work. Part of the machinery is devoted to transforming the energy-rich compounds from the blood stream into other compounds whose energy can be used directly by the contractile elements. These metabolic reactions will not concern us at this point, and we will restrict our attention to the structures which are directly involved in contraction. These structures are built out of protein molecules. During contraction some sort of physical re-arrangement takes place in the structure which causes it to shorten and do work against an external load. This large-scale shortening must be a reflection of events taking place at the molecular level, events associated with the interaction of protein molecules with the smaller molecules, probably adenosinetriphosphate (ATP), which supply the energy. Thus any understanding of the mechanism involved must be preceded by knowledge of the structure of muscle at the molecular level. Two principal techniques are available for acquiring such information. These are, electron microscopy and *x*-ray diffraction.

1. Electron-Microscope Observations

Electron-microscope studies on muscle (Hall, Jakus & Schmitt, 1946; Draper & Hodge, 1949; H. E. Huxley, 1953a) have shown that the structural proteins are organized into longitudinal filaments, of diameter in the range 50–100 Ångström units (1 Å = 10^{-8} cm.). These filaments form a very regular array across the myofibrils; they are spaced out a few hundred Å apart and, in life, the intervening region is filled with a dilute solution of soluble proteins and of salts, ATP, etc. When ultra-thin cross-sections of muscle are examined (H. E. Huxley, 1953a), some strikingly significant features of these arrays of filaments begin to emerge. In the dense, birefringent A bands, two different types of parallel filament are present. The thicker ones, which have a diameter about 110 Å, are arranged in simple hexagonal fashion, about 450 Å apart. In longitudinal sections these thicker filaments may be seen to extend from one end of the A band to the other. The thinner filaments, whose diameter is about 40 Å, are each located in a regular manner in between three thicker ones, so that each of the thicker filaments is surrounded by six thinner filaments; it shares these with its six nearest neighbour thick filaments, each of which also has a total complement of six thin filaments. A typical electron micrograph of the structure is shown in Plate II, fig. A (a) (together with a longitudinal section, Plate II, fig. A (b)); and Plate II, fig. B (a, b) shows the arrangement schematically. In the centre of the A band, in the somewhat less dense H zone only the primary array of thicker filaments is observed. In the I bands only the thinner filaments are seen.

These observations led to the obvious suggestion that striated muscle is built up of two overlapping sets of filaments, one set extending in either direction from the Z line to the boundary of the H zones, and the other set extending from end to end of the A band.

2. The Location of Actin and Myosin

Strong support was lent to the idea of the two different sets of filaments by the observation (Hanson & H. E. Huxley, 1953) in the phase-contrast light microscope that, when muscle fibrils are treated with solutions known to dissolve out the structural protein myosin, a large amount of material is selectively removed from the A band, so that the A-I boundary disappears and a sort of " ghost " fibril is left, consisting now simply of material stretching from the Z lines to the position of the edge of the former H zone. The H zone itself now has very low density indeed, but structural continuity must still exist across it, for the " ghost " fibrils do not fall apart, even when floating freely in salt solution. When these " ghosts " are examined in the electron microscope (Hanson & H. E. Huxley, 1953; Hasselbach, 1953), it is found that the thick filaments whose array formed the A band are no longer present, and only the thin filaments extending from the Z line to the H zone are left. It seems very likely, therefore, that the thicker filaments are largely composed of myosin, and thus we can distinguish between filaments of the two types not only on the basis of their diameters as seen in the electron microscope, but also on the basis of their composition.

This process can be extended a little further. The myosin extraction procedures leave behind the second principal structural protein, actin. Actin can be extracted by treating the myosin-free residue with 0.6 M-KI. When this process is observed taking place in isolated fibrils in the phase-contrast light microscope (Hanson & H. E. Huxley, 1954), the " ghosts " lose all their visible material between the Z lines and the H zones, leaving behind the Z lines themselves, still joined together by some backbone material giving structural continuity. These three stages of extraction are shown in Plate II, fig. C.

Thus a remarkably simple picture emerges of two overlapping sets of filaments, built up principally of the two separate structural proteins myosin and actin, with myosin in the thicker filaments and actin in the thinner ones. The observed ratio (2:1 or 3:1) of myosin to actin in muscle is in approximate agreement with the amounts one would expect from the lengths and diameters of the two types of filament.

3. Interference Microscope Observations

The ideas discussed above have recently been put on a more quantitative basis by interference microscope measurements of the relative amounts of protein present in the different

bands of muscle, and of the changes in those amounts which take place during myosin extraction (H. E. Huxley & Hanson, in preparation). It is found that the " extra " material in the A band accounts for about 54% of the total protein of the myofibril. When the myosin extraction procedure is carried out, all this extra material is removed, together with a small part of the material of the I-band filaments, so that in all about 60–63% of the material of the fibril is extracted. Large-scale biochemical analyses of the same process have also shown that about 60–70% of the myofibrillar protein is removed, and have further demonstrated that myosin accounts for only about five-sevenths of this; i.e., that myosin makes up about 45–50% of the fibrillar protein and that a further 15–20% of the fibrillar protein known as Szent-Györgyi's X protein (Szent-Györgyi, Mazia & Szent-Györgyi, 1955) is extracted along with the myosin. These results prove that at least four-fifths of the myosin is present as the extra material of the A band (i.e. the thick filaments); and, in the author's view, all of the myosin, together with about half of the X protein, probably makes up the 54% of the myofibrillar material present as " A substance ", the rest of the X protein coming from the I-band filaments (probably denatured actin). The alternative view, that one-fifth of the myosin is present in the I-band filaments, and that all the X protein is present in the A band (or an intermediate model, in which some X protein is present together with some very small amount of myosin in the I-band filaments), is of course logically permissible, but it seems inherently less plausible.

4. A Model Structure for Muscle

The appearance of muscle in the light microscope arises, then, in a remarkably direct manner from the way in which the structural proteins are arranged. We have the denser parts of the A band where the two sets of protein filaments overlap, the less dense central part of the A band (the H zone) where there are only myosin filaments, and the still lower density of the I band where there are only actin filaments. Furthermore, the microscopically visible changes in these bands, which take place during contraction and during stretch, can now be correlated with changes on molecular level. It has already been described how, during contraction, the I bands decrease in length, the A bands remaining at constant length until the Z lines are drawn into contact with the ends of the A bands at about 65% resting length (A. F. Huxley & Niedergerke, 1954; H. E. Huxley & Hanson, 1954). Further shortening then produces " contraction bands " in this region. When this process is studied in the phase-contrast microscope (by observing the ATP-induced contraction of isolated fibrils), it can be seen that the H zones become shorter at the same time as the I bands decrease in length; when the H zones have completely closed up (at about 85% resting length), further shortening gives rise to a dark line in the place of the H zone. When the muscle is stretched, the I bands increase in length, the A band again remaining at constant length, but now the H zone becomes longer by an amount equal to the increase in length of the I band. In the electron microscope, it can be seen that this longer H zone is still characterized by the absence of secondary filaments from it. It is quite clear, therefore, that both in contraction and in stretch we have a process in which the two sets of filaments slide past each other, the filaments of each set remaining at least at approximately constant length until the point is reached where they would have to crumple up for steric reasons. During stretch, this sliding process is simply brought about by the two sets of filaments being withdrawn from each other by the externally applied force. During contraction, the sliding must be brought about by internal forces; it is only natural to think of these in terms of obliquely oriented cross-linkages between the actin and myosin filaments; such cross-bridges have in fact been observed in the electron microscope, and they seem to occur with a regular spacing of 200–400 Å, but we have as yet no structural information that would help us to understand how they operate.

5. Low-Angle *x*-Ray Studies on Muscle

x-Ray studies on muscle (H. E. Huxley, 1953b), which in fact preceded a good deal of the work described above, have given results which are in very close accord with it. The low-angle *x*-ray diagram, which gives information about structural repeats in the range 10–1,000 Å, shows, in the first place, a small number of well-marked equatorial reflections (i.e. reflections from periodicities at right angles to the long axis of the muscle) which correspond to those expected from a hexagonal array of long rods spaced out about 450 Å apart. In living muscle, the intensities of the reflections correspond to those expected if the space between the rods is occupied by material of fairly uniform density. In muscle which has gone into rigor (a condition characterized by greatly increased rigidity and resistance to stretch, and associated with loss of ATP), the intensities (but not the spacings) of the reflections differ in a striking manner from the ones given by live muscle; the new intensities are compatible with the presence of a secondary array of long rods arranged parallel to the first, with each secondary rod now localized at points midway between three rods of the primary array. This interpretation was in fact put forward before the structure was seen in the electron microscope, so it does provide an important degree of independent confirmation. It was suggested, on the basis of the *x*-ray data, that the proteins actin and myosin might be organized into separate filaments, and that in living muscle, where ATP would dissociate the actin–myosin linkage, the secondary filaments would not occupy fixed positions relative to the primary ones, and so would only produce a more or less uniform background which would not affect the *x*-ray reflections from the primary array; in muscle in rigor, the secondary filaments would " crystallize out " in between the primary ones as actin–myosin links formed, and the *x*-ray diagram would change accordingly. It was further suggested that the formation of such cross-linkages would prevent the muscle from being stretched when in rigor if one of the types of filament was inextensible.

6. Axial Periodicities in the Filaments

Living muscle also gives a detailed pattern of axial reflections at low angles, corresponding to a repeat period of about 415 Å. Part of this pattern is also visible in dried muscle (Bear, 1945). No detailed interpretation of this diagram in terms of the internal structure of the filaments has been given, but very similar diagrams have been obtained from fibrous actin (Astbury & Spark, 1947). Indeed, it is noteworthy that a 400 Å axial period is often observed in muscle in the electron microscope, the periodicity being visible in both A and I bands. At present it is not clear whether the same periodicity extends

across the wide H zone in stretched muscle (the position is confused in muscle at resting length by other lines in the H region), but it would be only too likely that similar spacings would be present in both myosin and actin filaments if regular cross-bridging to specific sites were to occur. The most important feature, however, of the 415 Å *x*-ray spacing is that it does not increase when the muscle is stretched nor, it seems, is any decrease observed in spacing with isolated fibres which have been allowed to contract in ATP. This was interpreted in terms of inextensible actin filaments, and it will be obvious that this result and interpretation is in exact agreement with the more detailed model suggested by the later observations.

7. High-Angle *x*-Ray Studies on Muscle

At wider angles, the *x*-ray diagram also reveals significant structural regularities (Astbury, 1947). The reflections—a meridional arc at 5.1 Å and one on the equator at about 10.5 Å—are those characteristic for fibrous proteins with their polypeptide chains coiled up into the α-configuration (Pauling & Corey, 1951), the chain direction being along the length of the muscle. The diagram from muscle is a poorly oriented one. When live muscle is stretched, the diagram remains that of an α-protein. Exposure-time limitations have so far precluded the study of similar diagrams from striated muscle in the act of contraction, although diagrams have been obtained from muscle which has contracted and has been allowed to remain at the shortened length; these diagrams still show only the α-diagram. Astbury (1947) has examined the wide-angle diagrams by actively contracting *Mytilus* retractor muscle (smooth), but found only a rather more poorly oriented version of the α-diagram given by the same muscle at rest. The α-diagram seems to originate from the myosin component of the muscle, for purified fibrous actin does not give the α-type reflections.

The difficulty in using this type of *x*-ray information to elucidate the contraction mechanism is that, on the model we have at present—and that model seems substantially correct—only a small part of the structure (i.e. the cross-bridges) undergoes large changes at the polypeptide chain level; the rest of the structure (i.e. the filaments) moves but does not seem to need to undergo any drastic change in its internal configuration. The wide-angle *x*-ray diagram can give only an over-all picture of chain configuration, in which local changes in a small fraction of the whole will be very difficult to detect. The fact that dramatic changes do not occur in the wide-angle *x*-ray diagram is certainly in agreement with the ideas we now have about how the structure contracts, but it does not help us to understand how the structure is made to contract.

8. Conclusions

Muscle is a structure whose working parts are built up largely from two proteins, actin and myosin. These two structural proteins appear to be organized into separate filaments. Neither type of filament extends continuously along the length of the muscle, although there is a continuous backbone structure of unknown composition and very low density. The filaments are organized into a succession of separate arrays, each array containing filaments of one type arranged in register and overlapping with the next array of filaments of the other type, also arranged in register. This system gives muscle its banded appearance. The thicker filaments contain myosin and are restricted to the A band, where they give rise to high density and birefringence. The thinner filaments contain actin and extend on either side of the Z lines, across the I bands, and into the A bands, where they interdigitate with the myosin filaments up to the boundaries of the H zone. In the H zone (at the centre of the A band), there are myosin filaments but no actin filaments. When the muscle contracts, or is stretched, the two arrays of filaments slide past each other, and the changes in the microscopically visible appearance are those which we should expect. The directed sliding motion during contraction is presumably brought about through the mediation of oblique cross-links between the filaments; such cross-links are the structural expression of the biochemical interaction between actin and myosin, and as ATP can affect that interaction, and may itself be enzymically dephosphorylated by myosin, so ATP must interact with the cross-linkages and enable the two types of filament to " crawl " past each other.

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THE THERMODYNAMICS OF MUSCLE

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1 Resting muscle
2 Heat production during muscular contraction
  a Measurement and temporal resolution
  b Relationship to shortening and work done
3 Heat production during recovery
References

1. Resting Muscle

Resting muscle under constant conditions liberates heat at a constant rate. At 20° C., with sufficient oxygen, the heat production of a frog sartorius is about 7 to 10 × 10^4 ergs/g. × min. (1.7 to 2.4 mcal./g. × min.). This is increased, or decreased, about 2.5 times by a rise, or fall, of 10° C. If human muscles are similar, the 30 kg. of muscle of a normal man, entirely at rest at 37.5° C., would give out about 300 cal./min., requiring 60 ml. O_2/min., which is a substantial fraction of the basal metabolic rate. This heat is presumably an accompaniment of the functional activity by which the biological and chemical organization is continually renewed against dissipative forces, and the internal medium is maintained against electro-chemical and concentration gradients. If oxygen is absent, the resting heat rate is reduced to about one-half, and the products of anaerobic metabolism, serving the same function, accumulate. If oxygen is readmitted before too long, recovery occurs with an extra liberation of heat: but if anaerobic conditions continue, irreversible damage is caused.

Two curious facts are known about the resting heat production of muscle: it is greatly increased by raising the external K-ion concentration (Solandt, 1936), and substantially increased by keeping a muscle under tension (Feng, 1932; v. Euler, 1935). The effect described by Solandt is probably related to the surface depolarization of the fibres and may provide a clue to the connexion between excitation and contraction. The effect observed by Feng may possibly be due to the activation, by mechanical stretch, of enzymes forming part of the protein structure.

A resting muscle under a small initial load has " rubber-like " elasticity—it shortens when warmed, lengthens when cooled. Conversely, when such a muscle is stretched, or released, its temperature rises, or falls: under greater initial loads the opposite effects are found, the structures which become taut at greater lengths have normal, not rubber-like, elasticity. These " thermo-elastic " effects are reversible, and are of a purely physico-chemical nature. They are more complicated in muscle than in other elastic bodies, they seem to be related not to the specific contractile mechanism, but only to the structural material in which that mechanism is manifested on stimulation (Hill, 1952).

2. Heat Production during Muscular Contraction

a. Measurement and Temporal Resolution

The heat production of muscle is chiefly of interest in connexion with active contraction caused by stimulation. The mechanical response of active muscle is easy to record; indeed too easy, for very inadequate methods have been used and the older physiological literature is burdened with the results of experiments which depended more on the properties of levers than on those of muscle. Not till comparatively recently have adequate methods of mechanical recording been adopted. The only other immediate signs of activity, following stimulation, are (i) the propagated electric change and (ii) the heat production. The electric change is associated with excitation and is discussed elsewhere.[1] The heat production is a sign, and a measure, of the chemical and physical events which accompany, or follow, contraction (Hill, 1950b).

The thermo-electric method is the only one which is sensitive and quick enough to measure, and to give the required temporal resolution of, the temperature changes associated with contraction. The principle of the resistance thermometer (today it might be the " thermistor ") has continually been suggested for the purpose. But this requires a current in the sensitive element, which produces heat that warms the muscle locally; and, when movement occurs, cooler parts of the muscle come in contact with the element, and grave errors are caused: the method can safely be disregarded (Hill, 1938, p. 152).

The thermo-electric method was first used more than a century ago by Helmholtz (1848): with three thermo-couples in series he was able to measure the heat produced in prolonged contractions of frog-leg muscles. Fick (1882) employed the method extensively, and Blix (1902) devised a special " thermo-galvanometer " which was modified later by Hill (1911) for his earlier work. The initial impulse to modern technique came, however, from Bürker in 1908 (see Hill, 1913), and since then methods have been continually improved (see Hill, 1937, 1938, 1949b; Abbott, Aubert & Hill, 1951). Today it is possible to record the rise of temperature throughout a contraction to within about 10^{-5} °C. and to resolve its time-course to within a few milliseconds. This is not the place to refer further to technical details, but it is a fact that most of our present accurate knowledge of muscle heat has come directly from improvements of instruments: and these, in the last 30 years (following Fenn, 1923), have been largely due to Mr A. C. Downing. The material, a living muscle, could not be coerced to fit the equipment: the equipment therefore had to be made to fit the material. It is only fair to add that the myothermic method has strict requirements as regards size, shape and structure of the muscles used with it: reliable results cannot be obtained with cardial and various other kinds of muscle.

The importance of measuring muscle heat is due to the fact that it provides the only sensitive and rapid method of following the internal physical and chemical changes that accompany and succeed contraction. Little can be learnt by simply measuring the total heat, which was the only thing possible before 1913: it is necessary to resolve that heat in time, during and after contraction, and to find out how its course and amount are influenced by mechanical conditions (such as the possibility of shortening) and by other circumstances (such as temperature or the presence or absence of oxygen). It is

[1] See Katz, p. 210 of this number of the Bulletin.—Ed.

true that the heat production, chemically speaking, is unspecific: even the most accurate description of it does not allow us to decide what exactly the underlying chemical events are, but it does provide a framework into which those must be fitted. During a single muscle twitch the heat production is about 0.003 cal./g. It occurs in two stages: (i) an initial very rapid one (starting before the mechanical response is visible, Hill, 1953b); this is associated with the process by which the muscle passes from a state of rest to a state of activity; (ii) one in which heat is liberated in proportion to the amount of shortening the muscle undergoes. Then follows a very slow heat production accompanying the process by which, in the presence of oxygen, recovery occurs and the chemical changes connected with contraction are reversed. No heat is liberated during relaxation (Hill, 1953c): if any appears to be, it is always found to be due to work—done by the muscle during previous shortening—being degraded into heat as the muscle lengthens.

During shortening, a muscle does mechanical work, according to the load lifted: this is in addition to the heat it gives out. Under the best mechanical conditions the work may amount to about 40% of the whole initial energy (heat + work) (Hill, 1939b). The recovery heat is about equal to the total initial energy (see below): which means, since the chemical changes of contraction are fully reversed in recovery, that the "efficiency" of recovery is about 50%—half the energy set free by oxidation is used for chemical synthesis, half is wasted as heat. The fact that no heat is produced during relaxation cannot be disregarded by those who provide chemical theories of how a muscle works: if they assume that some exothermic process occurs, such as the splitting of adenosine-triphosphate (ATP), they must introduce a corresponding endothermic process to balance it.

During a maintained contraction caused by a series of stimuli, as contrasted with a single twitch due to one stimulus, there is a continual production of heat as long as activity lasts. The rate of this heat production depends on the natural speed of the muscle. A rapid muscle produces a large amount of heat in a tetanus, a slow muscle produces a small amount. Raising the temperature of a muscle makes it quicker: so it produces more heat in a maintained contraction and fatigues sooner. During a long-continued contraction, a muscle becomes progressively slower and more economical (Bozler, 1930; Feng, 1931; Bronk, 1932). The range of speed of muscles is enormous: even in the striated muscles of vertebrates there may be a several hundred-fold variation of speed, and some smooth muscles are extremely slow. The slowest muscles can maintain contraction for a long time with little expenditure of energy, the fastest ones fatigue very quickly.

The suggestion has been made that muscle, during the state of activity due to stimulation, has thermokinetic properties like those of rubber, as resting muscle has. If so, it would shorten with an increase of entropy and a fall of temperature. In fact it does the opposite. However fast, or however slowly, it shortens, it always produces extra heat in proportion to the shortening, and that heat is independent of the work done. Moreover, if the tension of a muscle is released very rapidly during isometric contraction, before the contractile element of the muscle (as distinguished from the inert elastic element) has had time to shorten significantly, there is a sudden small rise of temperature (Hill, 1953a). This is what happens in a normal elastic body when the tension is released, but not in one with rubber-like elasticity. The resting muscle undoubtedly has rubber-like elastic properties: but stimulation transforms it rapidly into a different state, analogous to that of normal elastic substances. Similar thermo-elastic effects are found in muscle during the state of rigidity produced by treatment with iodo-acetate (Aubert, 1953): possibly this form of rigidity can be regarded as a state of activity permanently fixed without the usual continual flux of energy and chemical change. When an active normal muscle shortens, the work it does is derived from internal energy provided by chemical reaction, not from the heat of the surroundings with an increase of entropy (as in a gas expanding isothermally).

The magnitude and time-course of the heat liberated during (as distinguished from after) a contraction is unaffected by the absence of oxygen: the recovery process and the use of oxygen come later (D. K. Hill, 1940a). This is a good example of the kind of evidence which the myothermic method, because of its speed and sensitivity, can provide. It is hard to imagine another method by which it could be established so simply that the chemical processes during a single short contraction are entirely non-oxidative.

b. Relationship to Shortening and Work Done

In a muscle twitch, energy E is liberated in three forms: (i) A, heat of activation (i.e. of the process by which transformation from rest to activity is brought about); (ii) heat of shortening ax, where x is the amount of shortening and a is a constant; and (iii) mechanical work W. In a tetanic contraction the heats of activation due to successive stimuli are summed together and become the heat of maintenance. In the equation (Hill, 1949a, 1950b)

$$E = A + ax + W$$

the three terms on the right are independent of one another. This is rather remarkable, for one might have expected either that work would be done at the expense of some of the heat, or that its performance would lead to the production of more heat. Apparently when a muscle, shortening, meets a load, it provides extra energy exactly equal to the work it has to do, without any effect on the heats A and ax. No explanation can be given at present of this apparently fundamental property of the contractile mechanism.

The constant a of the heat of shortening is interesting. It has the dimensions of a force, and in all the muscles examined is about 400 g.wt./cm.[2] of muscle cross-section (Hill, 1938; Abbott, 1951; Abbott & Lowy, 1955). It occurs also in the equation

$$(P + a)(v + b) = \text{constant}$$

defining the relation between the speed of shortening v and the load P.[2] Here b is a constant depending on the intrinsic speed of the muscle; b is about one-quarter of the maximum speed of shortening under zero load. Why these simple and rather exact relations exist is at present unknown, but an even odder one has been noted. When a muscle is maintained in steady isometric contraction its heat production settles down to a nearly constant rate. This heat rate, in mechanical units, is approximately equal to ab: in words, the heat produced in maintaining a contraction at constant length is about equal to the extra heat of shortening at velocity b (Hill, 1939b, p. 449). This is a very peculiar coincidence, if it is a coincidence, for the two quantities might have been altogether different. Possibly we are being very stupid in not seeing what all these relations mean in terms of contractile mechanism.

[2] See also Wilkie, p. 177 of this number of the Bulletin.—Ed.

The applications of thermodynamics generally involve the second law, but with muscle it is seldom possible to employ thermodynamical reasoning in this sense. The free energies of the chemical reactions which are known, or believed, to take part in contraction can be calculated: they are about equal to their total energies. In principle, therefore, a muscle might turn a large fraction of its chemical energy into mechanical work; in fact, however (considering the whole cycle, initial and recovery), it cannot achieve a " mechanical efficiency " of more than 20–25%. Frequently it has been suggested that a muscle is a heat engine of some kind: and when it is objected that a muscle fibre works at a nearly uniform temperature, and cannot have large differences of temperature inside it, speculation has sometimes run riot about micro-sources and micro-sinks [3] of heat, regardless of the miraculous thermal insulation these would require located so close together in so small a space. The concept of entropy has been usefully applied to the elastic properties of resting muscle: but it is clear that an active muscle is not an entropy machine (like stretched rubber or compressed gas), for if it were, there would be a fall of temperature when external work was done, whereas in fact the temperature always rises. A whole family of theories—osmotic, surface tension, elastic, etc.—are killed by the rude fact that no authentic fall of temperature has ever been observed, even momentarily, at any stage of contraction or relaxation.

3. Heat Production during Recovery

The production of heat during recovery in the presence of oxygen can be measured in three different ways: (i) after a single short contraction; (ii) by comparing the total heat from a series of twitches in oxygen with that from a similar series in nitrogen, and (iii) during the steady state which sets in after a few minutes of continued stimulation at moderate frequency (Hill, 1928; Bugnard, 1934; Hill, 1939a; D. K. Hill, 1940b). During recovery following a single contraction, the rate of production of heat rises quickly to a maximum and then slowly falls to zero. A steady state, however, takes some minutes to be reached and then, provided that the supply of oxygen is sufficient, can be kept up for a long time. In all three cases the recovery heat is about equal to the initial (heat + work). Another method leads to the same result. A muscle in nitrogen is stimulated by a series of shocks, and the total heat is measured as in (ii) above. Then, when this heat has been dissipated and the deflexion is steady again, oxygen is gently admitted: a large deflexion occurs from which the recovery heat can be calculated; again it is about equal to the initial energy. All these phenomena have their counterparts in human muscular exercise: indeed experiments on isolated muscle and on human muscular exercise have acted and reacted with each other to build up the concepts (i) of " oxygen debt " after hard exercise, (ii) of the " steady state " during moderate exercise, and (iii) of the " oxygen requirement " of a given effort, measured partly during and partly after the exercise itself.

One peculiar by-product of the myothermic method has been its use to measure the rise of osmotic pressure during muscular activity in the absence of oxygen. The liberation of lactic acid, the splitting of phosphate compounds, etc., lead to an increased molar concentration in the water of the muscle. This causes a fall of vapour pressure which, in an isolated muscle in nitrogen, results in a distillation of water over on to the muscle. The heat of condensation of this water leads to a proportional rise in the apparent rate of heat production. The fall of vapour pressure so measured is greater than can be calculated from all the chemical changes known to occur, if one assumes that the precursors (e.g. creatine phosphate and ATP) of the substances produced were themselves osmotically active. These precursors, therefore, must exist in a combined form in resting muscle, producing no effect on the vapour pressure until they are split (Hill & Kupalov, 1930; Hill, 1950a). Incidentally (i) the admirable method which Baldes (1934) devised for measuring osmotic pressure in very small samples (down to 0.1 mg.) of solution (Fox & Baldes, 1935) was derived from this unexpected outcome of muscle heat measurements, and (ii) similar osmotic changes are found after hard exercise in human muscle (Margaria, 1930).

[3] A "source" is a region of higher temperature than its surroundings; a "sink" is of lower temperature. Thus heat flows from "sources" to "sinks".—ED.

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THE MECHANICAL PROPERTIES OF MUSCLE

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1 Isometric contraction: the tension–length curve
2 Isotonic contraction
3 Dynamic performance with various types of load
4 Twitch and tetanus: the active-state curve
5 The characteristic curves of muscle
References

Resting muscle is soft and freely extensible. On stimulation the muscle passes into a new physical state—it becomes hard, develops tension, resists stretching, lifts loads. This article deals with the problem of defining the new physical state in exact mechanical terms.

Most of the research on this subject has been performed on the striated muscle of the frog, and for the sake of clarity the conclusions will be presented in concrete form, as the results of a set of experiments performed with the apparatus shown in fig. 1. The muscle, a frog's sartorius (a), lies on an array of stimulating electrodes. Its pelvic end is clamped rigidly and its free tibial end is attached to a light duralumin lever (b) pivoted on ball-bearings. The position of this lever, and thus the length of the muscle, is recorded by a photoelectric arrangement which is not shown. Forces may be applied to the muscle by loading it (c) (the load is applied near the fulcrum in order to reduce the effects of inertia); and the length of the resting muscle adjusted by moving the stop (d). For some purposes it is necessary to have an electro-magnetic stop (e) which can be removed at any moment during the contraction. Instead of recording length changes in the muscle, one can record tension changes by means of the transducer (f). This is a small valve (RCA 5734) whose anode can be moved from outside, thus converting changes of tension into changes of voltage.

Twitch and tetanus. If the muscle is stimulated by a single short electric shock it responds by a " twitch ", i.e., a phase of contraction followed by a phase of relaxation. If the stimulus is repeated before the first response has had time to die away, the second response becomes fused on to the first. If the stimulus is repeated regularly at a high enough frequency (say, 30/sec. in frog sartorius at 0° C.), individual responses can no longer be detected, and the muscle shows a smooth, maintained contraction, or " tetanus ". Clearly it is much simpler to examine the mechanical state of the muscle during a tetanus than during the transient twitch. The exact relationship between twitch and tetanus will be dealt with later.

1. Isometric Contraction: the Tension–Length Curve

If the muscle is held at fixed length (i.e., attached directly to the transducer (f) in fig. 1) and tetanized, it responds by developing tension. If the resting muscle is set at various different lengths before stimulation, the tension developed is seen to be a function of muscle length, as shown by the upper curve in fig. 2.

Tension development is greatest when the muscle has about the same length as it had in the animal's body, which was 31 mm. in this case. Resting muscle is an elastic body, partly because of the connective tissue which it contains. The stress–strain curve for the resting muscle is shown by the lower curve in fig. 2.

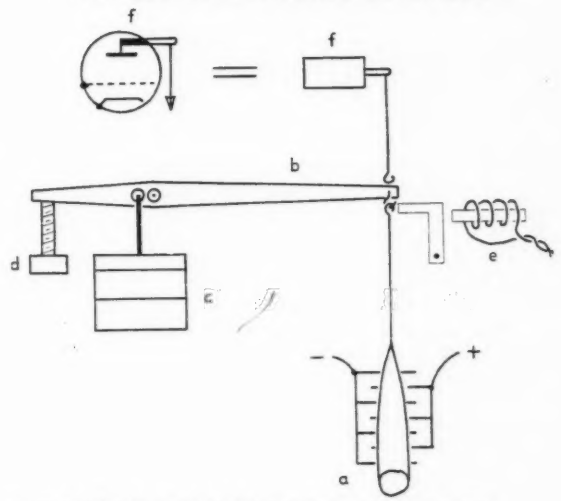
The series elastic component. There is a great deal of evidence (see Wilkie, 1954, p. 308 for references) that muscle consists of two components in series. The " contractile component " is the part which is altered by stimulation and is capable of active tension development and shortening. In series with it is an undamped passive " elastic element " through which the contractile component has to transmit its force to the muscle tendon.

The separate existence of these two components is perhaps most clearly demonstrated by the type of experiment illustrated in fig. 3 (Wilkie, 1956).

The experimental arrangement is shown in fig. 3a. The muscle is stimulated either by a single shock or by a series of shocks, but is prevented from shortening by the electromagnetic stop. It therefore develops tension isometrically. At a chosen moment the stop is withdrawn and the subsequent movement of the lever is recorded (fig. 3b). The movement shows two distinct phases—a rapid, almost vertical up-stroke due to the sudden shortening of the undamped series elastic component, followed by a much slower phase which is the shortening of the active contractile component.

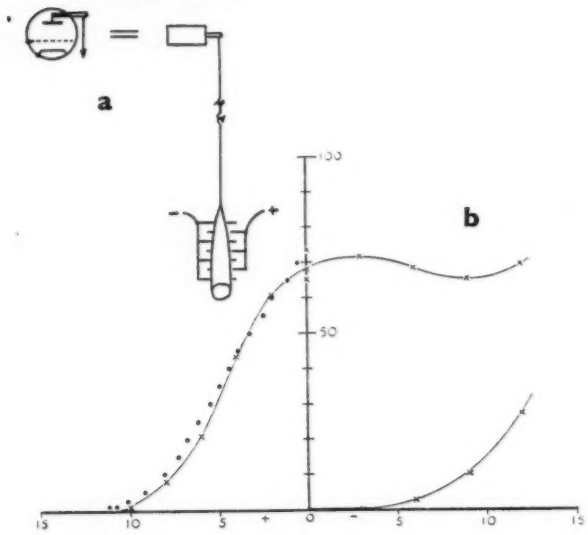
The amount of sudden shortening depends on the difference between the load on the lever and the isometric tension in the

FIG. 1. DIAGRAM OF APPARATUS FOR MEASURING MECHANICAL PROPERTIES OF MUSCLE



a: muscle (frog's sartorius) lying on stimulating electrodes
b: duralumin lever
c: muscle load
d: movable stop
e: electro-magnetic stop
f: transducer

FIG. 2. TETANIC TENSION-LENGTH CURVE, IN STIMULATED MUSCLE (UPPER CURVE) AND STRESS-STRAIN CURVE FOR RESTING MUSCLE (LOWER CURVE)



Abscissae: muscle length (mm.)
Ordinates: tension (g.wt.)
●——● isotonic recording
×——× isometric recording

a: arrangement of apparatus

b: the zero of the abscissae is at the in-situ length of the muscle (31 mm. in this case). Three control determinations of the isometric tension developed at this length were made at various times during the experiment.

muscle at the moment of release. By repeating the experiment with different loads one can measure the amount of sudden movement corresponding to each load, and thus plot out the stress–strain curve of the series elastic component.

The result is shown in fig. 4. In this experiment not only has the load been varied but also the moment of release has been set at various times following a single stimulus. The different curves in the figure thus represent measurements of the stress–strain curve made at different moments during a single twitch. The fact that all the curves fall on top of one another indicates that the series component is an inert object which is present all the time, not one which is called into being as a result of the stimulus and which disappears with the disappearance of activity in the muscle.

However, it is not clear what is the anatomical location of the series elastic component. Part of it must reside in the tendinous filaments into which the muscle fibres are inserted. The remainder may be a property of submicroscopic structures within the fibres, e.g. the Z-line region of the I band (Szent-Györgyi, 1953).

The series elastic component has an important effect on the mechanical properties of the whole muscle, for it smooths out rapid changes in tension. Thus it often makes a nuisance of itself in experiments which are aimed at examining the mechanical properties of the contractile component; for, whenever the tension is changing, the length of the elastic component must be changing too. This change in length (which can be calculated from the stress–strain curve) must be subtracted from the change in length of the whole muscle in order to find the length change in the contractile component.

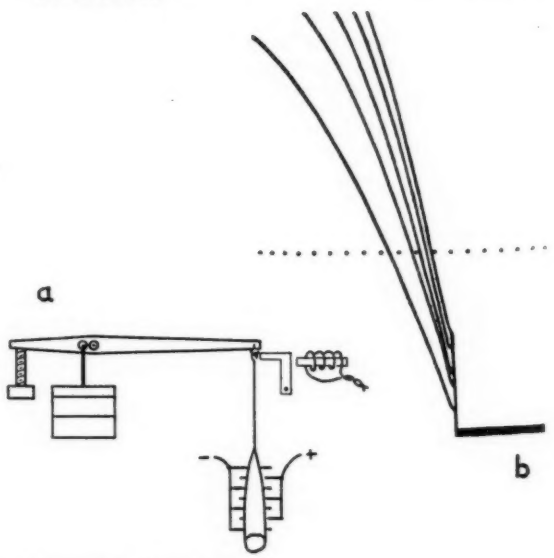
2. Isotonic Contraction

The need to correct for the series elastic component is largely eliminated by recording isotonically (fig. 5a). The load is supported by a stop until the muscle has developed enough tension to lift it. After that, the tension in the muscle remains constant throughout shortening, so the series elastic component must remain at constant length throughout each contraction. The inertia of the apparatus must be kept as small as possible by using a very light lever and by hanging the load very near to its pivot; for the effective inertia of the load is reduced in proportion to the lever ratio. The inertia can be still further reduced, if required, by hanging the load on a spring.

A set of isotonic shortenings against various loads is shown in fig. 5b. As the load is increased, three changes are apparent: (i) the latent period gets longer; (ii) the maximum amount of shortening gets less; and (iii) the initial velocity of shortening, i.e., the initial slope of the curve, also decreases.

i. The latent period is equal to the time taken for the muscle to develop isometric tension equal to the isotonic load. The larger the tension, the longer the time taken to reach that tension. Thus, if one were to plot latent period against isotonic load, the graph would be identical with the isometric tension–time curve.

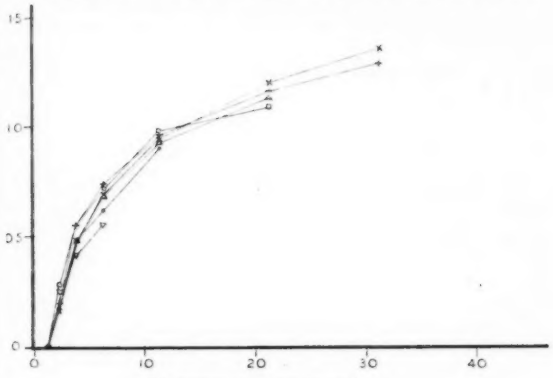
FIG. 3. DETERMINATION OF THE STRESS-STRAIN CURVE OF THE SERIES ELASTIC COMPONENT OF MUSCLE



a: arrangement of apparatus

b: Abscissae: time
Ordinates: shortening
The dots occur at 20-msec. intervals and are 2 mm. above the base line. Note that the curves read from right to left.

FIG. 4. STRESS-STRAIN CURVE OF THE SERIES ELASTIC COMPONENT OF MUSCLE, AT VARIOUS TIMES AFTER A SINGLE STIMULUS, USING EXPERIMENTAL ARRANGEMENT SHOWN IN FIG. 3



Abscissae: tension (g.wt.)
Ordinates: extension (mm.)
×——×: after 200 msec.
+——+: after 280 msec.
△——△: after 200 msec.
▽——▽: after 640 msec.
□——□: after 480 msec.
●——●: after 100 msec.

ii. The curve of maximum shortening against load is the same as the tension–length curve already determined from isometric observations. In fig. 2 the curve indicated by dots was obtained from isotonic experiments, the curve shown by crosses from isometric ones, on the same muscle.

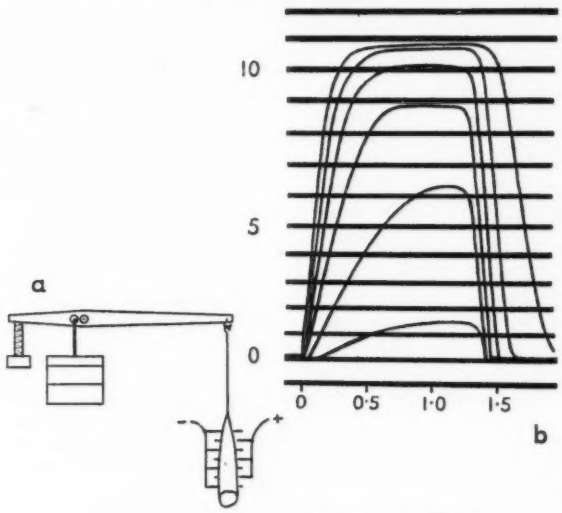
Put into more general terms this means that, when the shortening velocity of the contractile component is zero (i.e., when tension rise or shortening have reached their maximum), the tension in the muscle is a function of its length only; it does not depend, for example, on the initial conditions or on the route taken to reach final equilibrium. This statement needs some qualification; for there is evidence, at present incomplete, that it is strictly true only if the initial length of the muscle is not very much greater than the in-situ length.

iii. If one plots isotonic force against initial velocity of shortening, a curve of characteristic shape is obtained (fig. 6). The curve shows that even when there is no load on the muscle the velocity has a certain limited value. It does not become infinite as would that of an undamped elastic body. (The difference is shown clearly by the two phases of shortening in fig. 3b.) When the velocity is zero, i.e. under isometric conditions, the force is maximal. The line joining these two end-points is curved, not straight. This shows that, if the system is a viscous-elastic one, the viscosity must be non-linear.

However, there is other evidence, notably that from heat measurements (Hill, 1938), which indicates that the velocity is not limited by a passive internal viscosity in the muscle; it is much more probable that the chemical reactions which produce muscular energy are themselves controlled by the force on the muscle.

The shape of the force–velocity curve is more or less the same for muscles from many different types of animal. In all cases the maximum isometric tension is about the same—

FIG. 5. ISOTONIC CONTRACTION OF TETANIZED MUSCLE AT 0° C.



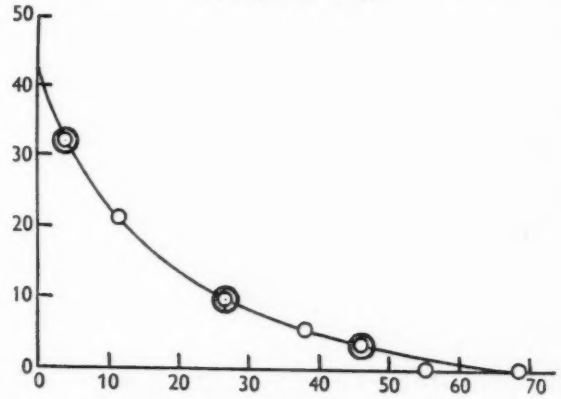
Abscissae: time (sec.)
Ordinates: shortening (mm.)

a: arrangement of apparatus
b: curves showing contraction at following tensions, reading from top to bottom: 1, 2.5, 5.0, 10.0, 20.0, 30.0 (g.wt.)

1 or 2 kg./cm.^{2} However, the velocity scale varies enormously between quick striated muscles (e.g. frog sartorius at 25° C.) and slow smooth ones (e.g. the retractor muscle of the snail pharynx).

Many algebraic equations can be fitted to the force–velocity

FIG. 6. FORCE–VELOCITY CURVE OF TETANIZED MUSCLE AT 0° C.



Abscissae: force (g.wt.)
Ordinates: velocity (mm./sec.)

Small circles: experimental points
Large circles: points "used up" in fitting the theoretical curve. Agreement between theory and experiment is significant only at other points on the curve.
Curve drawn from Hill's equation (see text, section 2).

curve. The most important of them is the one given by Hill (1938):

$$(P + a)(V + b) = (P_0 + a)b$$

where P = force, $V = dx/dt$ = velocity, P_0 = isometric tension, a and b are constants with the dimensions of a force and a velocity respectively. Arranging the equation in this form makes it clear that the force–velocity curve is part of a rectangular hyperbola. The equation can also be rearranged into the form $V = (P_0 - P)b/(P + a)$, which may be more evocative, for it shows that the velocity depends on the difference between the actual force acting on the muscle (P) and the maximum force which it could develop (P_0).

Not only does Hill's equation fit the experimental force–velocity curves of many different types of muscle; but also one of its constants (a) can be determined independently from either mechanical or thermal measurements, and the two estimates agree reasonably well.

It should be noted that, since P_0 appears in it as a constant, Hill's equation can be applied only near the flat top of the tension–length curve, where the muscle has about its in-situ length. Moreover, since the elastic component has been eliminated by recording isotonically, the equation applies to the contractile component alone.

3. Dynamic Performance with Various Types of Load

In the region of its length in situ, the mechanical behaviour of the tetanized whole muscle thus appears to be fully determined by the equation

$$V = (P_0 - P)b/(P + a) - d(f_1(P))/dt$$

where $f_1(P) = x$ is the equation of the stress–strain curve, known experimentally from fig. 4. This makes it possible to predict how the muscle will react when it is confronted by any specified mechanical system.

Inserting the appropriate relationship (imposed by the external mechanical conditions) into the above equation, the following cases have been worked out:

i. Isometric contraction; $V = 0$, therefore

$$(P_0 - P)b/(P + a) = d(f_1(P))/dt$$

i.e., the rate of internal shortening of the contractile component = rate of internal lengthening of the elastic component. The solution to this equation describes the way in which isometric tension rises with time. It is in good accord with experimental fact (Hill, 1938; Wilkie, 1950a).

ii. Imposed constant velocity (Levin-Wyman lever); V = constant (Hill, 1938).

iii. Inertia wheel; $P = M\,dV/dt$ (Hill, 1940).

iv. Inertia + constant force F;

$$P = F + M\,dV/dt.$$

This is the situation which confronts a muscle in the living body. The predicted result, which becomes oscillatory under certain conditions, is in good agreement with experimental findings (Wilkie, 1950a).

Although it is easy enough to write down these differential equations, it is often impossible to solve them algebraically, because they are non-linear. The result must then be obtained by computational methods or, much more simply and quickly, by building an electrical analogue circuit (Wilkie, 1950b). The contractile component is imitated by a battery in series with a non-linear resistor; the elastic component by a non-linear capacitance and the external load by appropriate inductances (= inertias) and batteries (= forces).

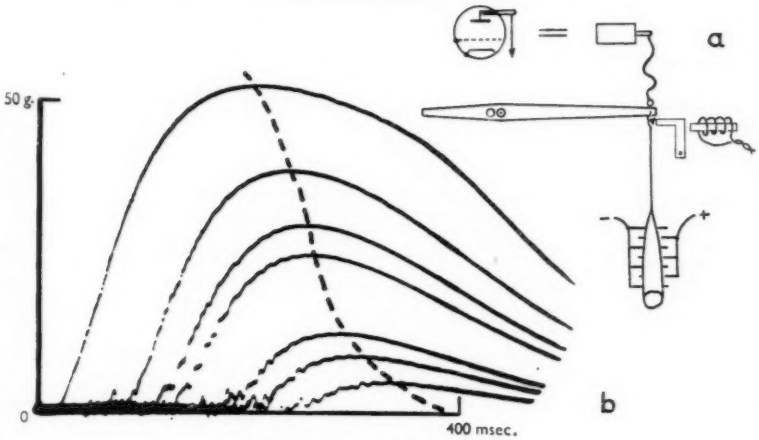
4. Twitch and Tetanus: the Active-State Curve

The physical state of a muscle is exactly the same during the early part of a twitch and during a tetanus. (After all, the muscle cannot know after the first stimulus whether or not other stimuli are coming.) However, the contracted state or "active state" does not last very long; and after only one stimulus there is not time for the isometric tension or the isotonic shortening to reach their full (tetanic) values. The exact way in which the active state appears and disappears has been intensively studied in recent years, for there is abundant evidence that many drugs act by altering the time-course of this process.

Definition of active state. The intensity of the active state at any instant is defined as "the isometric tension which the contractile component can develop (or just bear without lengthening) at that instant". The tension in the whole muscle follows a much slower time-course because of the series elastic component.

The onset of activity begins very soon after the stimulus. The first change in mechanical properties (at 0° C.) can be detected after 3 msec. (Hill, 1951b); spontaneous tension development after about 12 msec. (Abbott & Ritchie, 1951); and isotonic shortening after about 20 msec. (Hill, 1951a). Since isotonic shortening begins at its full maximum speed, one may conclude that by this time the active state has already reached its full intensity.

FIG. 7. ACTIVE-STATE CURVE OBTAINED FROM EXPERIMENTAL TENSION–TIME CURVES



Abscissae: time (msec.)
Ordinates: tension (g.wt.)

a: arrangement of apparatus

b: curve (interrupted line) drawn through peaks of tension–time curves shows decline in intensity of active state (as described in section 4)

The plateau of activity. Activity remains at full intensity for an appreciable time after the stimulus (40 msec. at 0° C., 10 msec. at 20° C.). During this period the muscle behaves exactly as though it had been tetanized (Macpherson & Wilkie, 1954). If the effect of the series elastic component is removed by a quick stretch of appropriate amount (Hill, 1949) or by sudden isotonic loading (Wilkie, unpublished, 1955), the muscle can develop, or bear, the full tetanic tension.

The decline of activity. The apparent duration of the plateau varies inversely with the sensitivity of the tension-recording apparatus employed. Thus, if a piezo-electric crystal is used instead of a transducer valve, the first decline from the plateau can be detected at 34 msec. (Ritchie, 1954a) instead of the 40 msec. quoted above. The falling phase of the active-state curve can be determined without this ambiguity by the method described by Ritchie (1954b). When the rate of change of tension in a muscle is zero, its elastic component must be at unchanging length. This occurs at the peak of an isometric twitch; and, since the total muscle length and the length of the elastic component are then both unchanging, the contractile element must also then be neither lengthening nor shortening. In this situation, the tension which it exerts (which is the same as the tension exerted by the whole muscle) must be equal to the intensity of the active state, according to the definition given above. By releasing the muscle at various instants after the stimulus, Ritchie obtains a set of twitch-like tension records (fig. 7b). The peak of each of them must lie on the active-state curve, which is thus indicated by the interrupted line in fig. 7b.

The apparatus is arranged as shown in fig. 7a. The (unloaded) lever is prevented from moving by the stop, and is attached to the transducer by a slack connexion. The amount of slack does not matter so long as it is greater than the amount by which the series elastic component is stretched at the height of contraction—that is, about 1.5 mm. (see fig. 4).

When the muscle is stimulated, it develops tension isometrically, but this is not recorded. When the stop is suddenly removed, the series elastic component shortens abruptly (as in fig. 3b) and the tension falls to zero. However, an active contractile component then redevelops tension, which is recorded as in fig. 7b. The later the release, the less tension redevelops.

The active-state curve is very easily influenced. Its falling phase is delayed by adrenaline, caffeine (Goffart & Ritchie, 1952), nitrate, bromide, iodide (Hill & Macpherson, 1954), quinine (Lammers & Ritchie, 1955), certain quaternary ammonium salts (Ritchie & Wilkie, 1956); also by previous stimulation (Ritchie & Wilkie, 1955), decrease in temperature (Macpherson & Wilkie, 1954), or increase in hydrostatic pressure (Wilkie, unpublished, 1954). These effects are probably all mediated at the surface of the muscle fibres (Hill & Macpherson, 1954).

5. The Characteristic Curves of Muscle

In earlier sections the mechanical condition of active muscle has been specified by four curves:

i. The stress–strain curve (fig. 4) of the series elastic component, $x = f_1(P)$;

ii. the tension–length curve (fig. 2b), $P_0 = f_2(x)$;

iii. the force–velocity curve (fig. 6), $dx/dt = f_3(P)$;

iv. the active-state curve (fig. 7b), $P_0 = f_4(t)$.

P = force, P_0 = isometric force, x = length, t = time, f_1, f_2, etc. are to be regarded purely as empirical functions defined by the experimentally determined shapes of the curves.

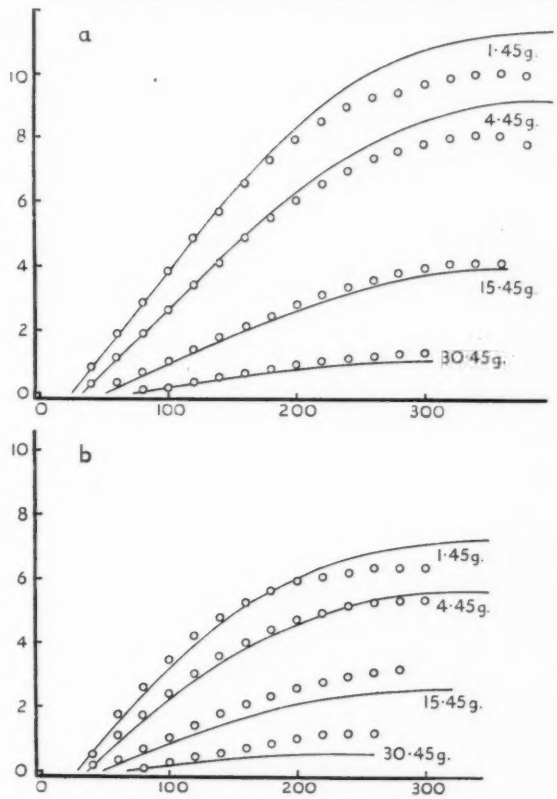
Curve i (cf. fig. 4) does not depend directly on the contractile machinery. Curves ii and iii (cf. figs. 2b and 6) seem to express the properties of the contractile proteins inside the muscle fibres. Similar curves are obtained from glycerol-extracted muscle fibres, activated by adenosinetriphosphate; and even from artificial threads of muscle protein which has been in free solution (see, for example, Weber & Portzehl, 1954).

In contrast, curve iv (cf. fig. 7b) arises from the mechanism by which the contractile machinery is switched on and off in response to changes of potential at the cell membrane.

Each of the curves gives only a partial view of the active muscle, for each of them is made by holding all but two of the parameters constant and observing the relationship between the remaining pair. How should one combine the curves when all the parameters are varying at once, as they may do in an actual contraction?

Shortening and the tension–length curve. The force–velocity

FIG. 8. THEORETICAL AND EXPERIMENTAL ISOTONIC TWITCHES AT VARYING TENSIONS, FOR MUSCLES AT TWO INITIAL LENGTHS (L_0 AND L_0–6)



Abscissae: time after stimulus (msec.)
Ordinates: shortening (mm.)
a: muscle length (L_0)
b: muscle length (L_0–6)
Circles: experimental curves
Lines: theoretical curves
The number on each pair of curves indicates the isotonic tension in g.wt.

curve, $dx/dt = f_3(P)$ can be written in algebraic form (Hill's equation):

$$dx/dt = (P_0 - P)b/(P + a)$$

Since P_0 appears in it as a constant, this equation applies only for small length changes in the region near the flat top of the tension–length curve. However, the equation can be modified to apply at other lengths by arranging that P_0 should vary with muscle length according to the tension–length curve:

$$dx/dt = (f_2(x) - P)b/(P + a)$$

This equation describes the full range of shortening of the tetanized muscle with fair accuracy (Abbott & Wilkie, 1953).

Dynamics of a single twitch. Hill's equation can be modified also to take account of the decline in activity following a single stimulus, by altering P_0 both as a function of time and of length:

$$P_0(x, t) = f_2(x).f_4(t)/P_0^*.$$

P_0^* is the original P_0 of Hill's equation, i.e., the tetanic tension at body-length, so the equation becomes

$$dx/dt = (f_2(x).f_4(t)/P_0^* - P)b/(P + a).$$

This equation can be tested by inserting experimentally determined values for P_0, a, b, and for the tension–length and active-state curves; then integrating to find how x changes with t for different values of P; i.e., one predicts the shapes of isotonic twitches against various loads. This work (Ritchie & Wilkie, 1955, 1956) is still in progress. Results so far show fair accord between theory and experiment, as illustrated in fig. 8: the curves show the results obtained when the equation is tested for two different initial lengths of muscle, as well as for several different loads. This equation may therefore be regarded tentatively as the general equation describing the mechanical properties of muscle; from it the various characteristic curves emerge as special cases.

An analysis of the mechanical changes along these lines should make possible a more penetrating analysis of the various factors which influence muscular contraction. Thus many drugs alter the active-state curve only; temperature change alters both the active-state curve and the force–velocity curve; while the tension–length curve is more or less independent of external influences. The curves thus appear to reflect separately the properties of separable parts of the contractile machinery.

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MUSCLE PROTEINS

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1 Extraction of the fibril proteins from rabbit
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Almost a century has passed since Kühne (1859) first began to study the protein components of skeletal muscle. He was followed by such well-known physiologists as Danilewsky, von Fürth and Halliburton, but, in reality, little progress was made until about 1930, when the newer physical methods—flow birefringence (von Muralt & Edsall, 1930), *x*-ray diffraction (Astbury & Dickinson, 1935)—were applied to the main globulin component, myosin. Whilst these stimulated a wider general interest, the more rapid development of the field since 1940 has depended less on the advent of new techniques than on several fundamental discoveries which resulted from simple observation. Amongst the latter one can cite the demonstration of Liubimova & Engelhardt (1939) that the adenosinetriphosphatase (ATPase) activity of muscle is largely associated with, and not separable from, the myosin itself—a discovery whose significance was at once acknowledged because of the growing belief at the time that the breakdown of ATP to adenosinediphosphate (ADP) and inorganic phosphate was closely associated with the act of contraction. Equally important was the discovery of Szent-Györgyi and co-workers in Hungary (1941–2, 1942, 1943), foreshadowed by the studies of J. Needham and others in Cambridge (Needham, Shen, Needham & Lawrence, 1941), that classical myosin is in reality a mixture of two proteins, myosin and actin, whose unique affinity for one another is profoundly influenced and modified by ATP. From the many-sided evidence accumulated in the last ten years, these two discoveries encompass all that seems basically essential in contractile phenomena—myosin acting as an ATPase, actin and ATP. Unfortunately, the evidence is descriptive rather than mechanistic, and in molecular terms we are still ignorant of the fundamental details of contraction.

Techniques for the isolation of myofibrils in a physiologically active state, i.e. capable of contraction, have enabled studies to be made of the contractile system freed from the many glycolysing enzymes which constitute the bulk of the sarcoplasm (Schick & Hass, 1949; Perry, 1951). Such studies are discussed more extensively by Dr Perry, but they show (Perry, 1953), as had already been surmised, that the fibrillar material is made up largely of myosin, actin and tropomyosin. The latter is slowly leached out by dilute borate buffer, in company with another component which appears to be a form of actin (Corsi & Perry, private communication). This behaviour makes us aware of the possibility that unknown components could also be lost from the myofibril during the isolation procedure; in addition, it must be emphasized that, at the histological and electron optical level, there do exist structures such as the Z line in the middle of the I band, or the thick filaments in the middle of the A (see Hanson & H. E. Huxley, 1955) which have not been chemically characterized.

The present discussion is intended to act as an introduction to other contributions, which, whether from the morphological or enzymic standpoint, deal with contractility as a property of organized structures; it will centre largely on the properties of myosin and actin in the extracted form, and less so on those of tropomyosin, whose function is quite unknown. The literature on the muscle proteins is not only vast, but often confusing, because there are so many bones of contention between different groups of workers. Space and clarity alike demand the presentation of an over-simplified picture, though a fuller treatment can be found in several books and reviews published during the last few years (Mommaerts, 1950; A. Szent-Györgyi, 1951; Weber & Portzehl, 1951; Needham, 1952; Bailey, 1954; Dubuisson, 1954; A. G. Szent-Györgyi, 1955; Morales, Botts, Blum & Hill, 1955; Perry, 1956b).

1. Extraction of the Fibril Proteins from Rabbit

All methods for the preparation of myosin employ coarsely minced pre-rigor muscle as starting material. If this is extracted for a short period with salt solutions (ionic strength ~ 0.5), very little actin is dissolved, particularly if the pH is maintained at a slightly acid reaction (pH 6.5) (Guba & Straub, 1943). The myosin is precipitated by diluting to ionic strength 0.04, thus removing most of the sarcoplasmic protein. Any actomyosin complex present in the precipitate can be centrifuged out after dissolving the true myosin at ionic strength 0.3 (Portzehl, Schramm & Weber, 1950). In actual fact, many different " recipes " exist for the preparation of pure myosin, though the criteria of purity for such an asymmetric protein are most difficult to establish. The choice of method should be guided by simplicity and aim, and only molecular studies require the use of pure material.

The preparations of both actin and tropomyosin are novel in the use of minced muscle that is initially extracted by an appropriate solution and then dried in organic solvents. For actin, the preliminary removal of some of the myosin is an advantage; and, before dehydrating, a wash to extract salt ions, which would prevent the final extraction of actin in its non-viscous form (so-called G actin). In contrast, the final extraction of tropomyosin is made by strong salt solution, followed by a well-defined purification procedure (Bailey, 1948). In both methods, the dehydration step causes denaturation of residual myosin, which would greatly complicate the method of preparation. Straub's original method (1942, 1943) for the preparation of actin gives material which does not entirely transform to the viscous form (so-called F actin), and modifications have been proposed by Mommaerts (1951) and Tsao & Bailey (1953).

These methods are not necessarily valid for all kinds of skeletal muscle. In fish muscle, for example, the differential extraction of myosin and actin is much more difficult (Connell, 1954), whilst tropomyosin can readily be obtained by direct extraction of wet muscle (see Hamoir, 1955).

2. General Properties of Fibril Proteins

a. Myosin

The salient properties of myosin arise out of its globulin character and its fibrous nature. At high rates of shear it exhibits double refraction of flow (Joly, Schapira & Dreyfus, 1955), a property much enhanced when actin is present. It is readily denatured by heat and by any kind of dehydration process—even freeze-drying. Films of myosin made by simple drying give, after stretching, the wide-angle *x*-ray pattern of α-keratin, and the same pattern is characteristic of muscle itself (Astbury & Dickinson, 1940). The long spacings given by striated muscle are not found in extracted myosin, and the available evidence suggests they arise from the actin component.

Well-purified myosin splits only the terminal phosphate group of ATP and related nucleotides, and is activated by Ca ions. Mg ions will neutralize the activation by Ca, and will not in themselves activate myosin if Ca is absent; however, actin-rich myosins are activated by Mg at neutral pH if K ions are absent. Any extended discussion of activation effects is not worth while, since, as Perry (1951) has shown, they do not necessarily hold for the enzyme as it exists in the structurally organized myofibril, whose enzymic properties are considered separately in the article by Perry in this number of the Bulletin (p. 188).

b. Tropomyosin

This is the only protein of the keratin–epidermin–myosin–fibrinogen (k–e–m–f) group which crystallizes[1] (Bailey, 1948). Perhaps its most striking property is its ability to aggregate in end-to-end fashion as salt ions are removed, owing presumably to an electrostatic attraction of one molecule for another. Such solutions, of course, are very viscous, but the intrinsic viscosity of sols containing only the depolymerized form indicates that the primary molecule or monomer is also very asymmetric. Tropomyosin, unlike myosin, is most resistant to denaturation, and its behaviour in this respect has been studied in some detail (Tsao, Bailey & Adair, 1951; Tan & Tsao, 1954).

Complexes of ribonucleic acid and tropomyosin, first characterized and crystallized by Hamoir (1951) from fish, have been obtained from both vertebrate and crustacean skeletal muscle, and from vertebrate heart and smooth muscle (Sheng & Tsao, 1954). In the case of skeletal muscle at least, the evidence favours the view that the nucleic acid is picked up as an adventitious impurity (Perry, 1953), although as much as 16% may be present in the crystals.

Actin

When actin is extracted from dehydrated muscle residue, the solution is limpid; the addition of salt, and especially of Mg ion, caused it to transform to a thixotropic gel (Straub, 1942; Feuer, Molnar, Pettko & Straub, 1948; Tsao & Bailey, 1953). The latter form, generally called F actin, appears to consist of long fibres in the electron microscope, and similar structures are found in muscle after the myosin is removed by differential extraction (Hasselbach, 1953; Hanson & H. E. Huxley, 1955). Preparations of actin, in common with isolated myofibrils, contain a small amount of nucleotide which appears to be dephosphorylated from ATP to ADP during the G → F transformation (Feuer *et al.* 1948; Mommaerts, 1952b). The monomer of actin, originally thought to be globular, is considerably asymmetric (axial ratio ~ 12) according to viscosity data (Tsao, 1953a). Its manner of participation in the formation of F actin is rather complex, and, as we shall see, open to different interpretations.

3. The Amounts of Myosin, Actin and Tropomyosin in Rabbit Muscle

Methods for the quantitative determination of proteins are beset with great difficulties, and the figures available must be accepted with this reservation (Bailey, 1954). In white rabbit muscle, the fibrillar protein comprises ~ 63% of the total intracellular protein, of which 44% is myosin and 15% actin (Hasselbach & Schneider, 1950); the rest, at least 3–4%, consists of tropomyosin. Since myosin is confined to the A band, these figures imply that the concentration of protein there must be quite high—perhaps over 20%.

4. Amino-Acid Analysis

The complete analyses which now exist for the three proteins (Bailey, 1954; Kominz, Hough, Symonds & Laki, 1954) provide no clue to their function. Tropomyosin and myosin contain large numbers of polar residues (63% and 57% of the total respectively), and the former contains no tryptophan, proline or —SH groups. All three proteins contain only traces of *N*-terminal groups reacting with fluorodinitrobenzene, yet *C*-terminal acids can be demonstrated in every case (Bailey, 1951; Locker, 1954). The —SH groups demonstrable in both myosin and actin amount to ~ 0.8–0.9% (as cysteine), and, in the case of myosin, some of these are essential to its ATPase activity.

5. Shape and Size of the Fibril Proteins

From studies of sedimentation and diffusion, light-scattering, flow birefringence, viscosity or osmotic pressure, we can derive information on the shape and size of protein molecules. Some of the data have been collected in Table I, which reveals that estimates may vary according to the worker and the method. Our present discussion will be confined to myosin and actin only, whose dimensions must ultimately be relevant in explaining the mechanism of contraction.

a. Myosin

From the work of Weber's group (see Weber & Portzehl, 1951) it has been widely accepted from sedimentation (*s*), diffusion (*D*) and osmotic pressure, that the particle weight of myosin is 850,000, and its dimensions 2,300 × 100 Å. Light-scattering (see Mommaerts, 1954) has suggested a shorter molecule (1,500 Å), whilst other values of *s* and *D* (e.g. Parrish & Mommaerts, 1954) have indicated a smaller particle weight. Some of the uncertainty has been removed by Laki & Carroll (1955), who find that fresh myosin has a low *s* value

[1] Recently, however, a new structural protein from adductor muscles, distinct from mammalian tropomyosin, myosin or actin, has been obtained in beautifully crystalline form, and this too gives a wide-angle α-pattern (Bailey, 1956).

at 5°, which increases on standing at room temperature, at first reversibly, and later irreversibly. The data indicate a particle weight of 500,000, suggesting that the Weber myosin was a stable aggregate of this smaller particle.

Now when trypsin acts for a short time on myosin, two different kinds of particle can be isolated which account for nearly all the nitrogen of the original protein. One of them, an albumin (H-meromyosin), possesses all the ATPase activity of the parent molecule, whilst the other, L-meromyosin, retains some of the globulin properties of myosin itself (see A. G. Szent-Györgyi, 1955). The particle weights of the proteins (232,000 and 96,000 respectively), together with their proportions (57% and 43%), suggest that the minimal particle weight of myosin must contain 1 mol. of H-meromyosin and 2 mol. of L-meromyosin. This weight of 425,000 is again about half that of Weber myosin. It thus seems that myosin, like actin and tropomyosin, can undergo aggregation, and that the weight of the primary particle is less than 5×10^5; moreover, since s for myosin is less than that of H-meromyosin, hydrodynamic considerations demand that in the native proteins the meromyosins lie end to end, giving a length of 1,700 Å (Lauffer & A. G. Szent-Györgyi, 1955).

Recent observations tend to show that in its ultra-structure myosin is a most unusual protein. By disaggregation in neutral urea solutions Tsao (1953c) had obtained a fraction with a molecular weight of only 16,000. More recently, A. G. Szent-Györgyi (1955) has reported that L-meromyosin in urea depolymerizes to units of even lower molecular weight (4,600), whilst H-meromyosin is unaffected. Whatever the significance of these findings for the explanation of contraction, they open up new fields in our knowledge of protein structure.

b. Actin

Actin possesses such unusual properties that it stands in a class by itself. It exists in a non-viscous form which, by addition of cations, especially Mg, aggregates into fibres capable of specific interaction with myosin. Values for the particle weight of G actin were at variance (Table I), until Tsao (1953a) obtained indications from osmotic pressure that, according to conditions, two forms of G actin can exist, a monomeric and a dimeric. This conclusion was also supported by applying G. Weber's polarization-fluorescence method (1952) to actin which had first been labelled with a fluorescent group. Chelating reagents and polyphosphates favoured the formation of the monomeric form (particle weight 70,000; cf. 60,000 (Mommaerts, 1952a)), and Tsao suggested that dimerization might be effected through a Mg ion acting as a bridge between two nucleotide prosthetic groups.

Tsao (1953b) also found that only dimeric actin could transform to F actin, and, most unexpectedly, that the fluorescence method was unable to distinguish between the one state and the other. At its face value, this finding may imply that the dimer units are spatially fixed in the actin aggregates, but free to rotate around their long axes, rather like a string of cylindrically-shaped beads. Even so, one might legitimately expect that the addition of myosin to F actin, giving as it does a system of such high and anomalous viscosity, would produce a static polymer, in which all rotation is quite restricted; instead, the actin was instantaneously converted to the monomer form, and could be reconverted to the dimer by addition of ATP.

Whilst the interpretation of the effects observed in polarization-fluorescence are somewhat equivocal, Chi & Tsao (1954)

TABLE I. SOME PHYSICAL PROPERTIES OF THE MUSCLE PROTEINS

Physical property	Tropomyosin (monomer)	G actin	Myosin	H-meromyosin	L-meromyosin
Sedimentation constant ($\times 10^{13}$)	2.6[a] ($c = 0.6$)	3.2[b]	5 → 8.2[c] (see text)	6.96[d]	2.86[d]
Diffusion constant ($\times 10^{7}$)	2.4[a] ($c = 0.6$)	2.5[b]	1.0[c]	2.91[d]	2.87[d]
Particle weight:					
s and D	—	150,000[b] (? dimer)	500,000→ aggregates[c]	232,000[d]	96,000[d]
Light-scattering	55,000[f]	57,000[g] 120,000[h] (? dimer) 80,000[j]	700,000[k]	—	95,000[e]
Osmotic pressure	53,000[l]	74,000[m] 140,000[m]	840,000[n]	—	—
Polarization-fluorescence	52,000[p]	70,000[m] 140,000[m] (dimer)	—	—	—
Intrinsic viscosity	0.523[l]	0.21[m] 0.5[m]	2.0[q]	0.28–0.36[d]	1.0[d]
Estimated length (Å)	400[l]	290[m]	1,700 (see text)	435[d]	550[d]
Estimated thickness (Å)	15[l]	25[m]	—	15[d]	25[d]

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have recently reported, after extending the earlier osmotic measurements, that " F-actin exists primarily in the dimeric rather than the polymeric form commonly accepted " and that " there is no indication that actin and myosin interact in such a way as to form giant complexes or co-polymers ". These results are certainly a challenge to the more conventional (though reasonable) views which regard F actin as a rigid polymer, combining with myosin to give an anastomosis of particles which is broken up or dissociated by ATP; such an explanation accounts nicely for all the macroscopic properties of the system, but, again, runs counter to the further observations of Blum & Morales (1953) that actomyosin particles (myosin B) by light-scattering techniques show no change in average particle weight on addition of ATP. Perhaps we shall need to invoke long-range forces to account for some of the properties of the myosin–actin–ATP system, but, however that may be, the results reveal a lability in the actin component, which might also be inferred from the way in which the actin particles of the I band fold up inside the A band during contraction (see Hanson & H. E. Huxley, 1955).

6. The Interaction of Myosin, Actin and ATP and its Bearing on Contraction and Relaxation

The study of model systems by many different workers, amongst whom must especially be mentioned Weber and his school in Germany, Szent-Györgyi and Bozler in the USA, Marsh and Bendall in England, has shown that ATP possesses two actions, one associated with contraction, and one with a " plasticizing " action which allows of passive relaxation. The two effects are necessarily opposed, the latter predominating if splitting of ATP is inhibited. The analysis of the effects in the case of models such as the glycerated fibre or oriented actomyosin thread is most complex, and entirely dependent on the ionic milieu, the temperature, the activating cation, the concentration of ATP, and the presence or absence of the relaxing factor discovered by Marsh (1952). We cannot as yet satisfactorily explain the duality in the action of ATP, but an analogy appears to exist in the behaviour of extracted actomyosin under certain conditions.

First of all, at low salt concentrations ($I < 0.2$),[2] actomyosin exists as a gel or a precipitate, which in presence of Mg ion and ATP exhibits an intense synaeresis which may increase the protein concentration from $\sim 2\%$ to 50% (Szent-Györgyi, 1941–2). This action is comparable to the behaviour of model systems when they contract or exert tension, and, wherever splitting has been measured, ATP (or certain other related nucleotides) is always broken down. Inorganic pyrophosphate (PP), which is not a substrate for ATPase (Bailey, 1942), cannot be substituted for the nucleotides.

When actomyosin is brought into solution at higher salt concentrations (e.g. 0.5 M-KCl), the effect on it of ATP is visibly different: it causes an instantaneous drop in viscosity, which rises again only when ATP is completely hydrolysed. Hydrolysis itself, however, plays no part, for the viscosity diminution can also be induced by PP, and this time is permanent. This plasticizing action of ATP on actomyosin sol is closely related to its effect on the models if by any means splitting is inhibited; they become less resistant to stretch, and behave more like physiological muscle in its relaxed state. Moreover, the plasticity can be induced by inorganic PP.

In a study of the interaction of myosin, actin and ATP in solution, Bailey & Perry (1947) showed that, as the ATPase centres of myosin were blocked by —SH reagents, the viscosity effects observed by adding actin to myosin, or of ATP to actin and myosin, were progressively abolished. Such experiments indicate that at high ionic strength, at least, the areas responsible for the interaction of actin and myosin, whatever its nature, are localized in the ATPase centres. The interaction is best described as an equilibrium:

$$A + M \rightleftarrows AM,$$

in which the back reaction is favoured by addition of ATP or PP, or even by greatly increasing the salt concentration, or lowering the temperature (Laki, Spicer & Carroll, 1952).

The mechanism by which ATP exerts its dual action is not known. A clue is given by the studies of Weber and his school on models, and by those of Perry (1956a) on the isolated myofibril, viz., that ATP added beyond the threshold optimum for splitting will itself inhibit splitting and favour relaxation. It seems probable (see Perry, p. 188 of this number of the Bulletin) that the real substrate for splitting is a Mg-ATP complex, towards which ATP is a competitive inhibitor (Perry, 1956a)—an antagonism also observed, interestingly enough, in the hydrolysis of PP by inorganic PPase (Bailey & Webb, 1944). As a working hypothesis, one may suppose that polyphosphate compounds combine with myosin in two ways: one, the relaxing position, which involves combination for the most part only with the PP group, and the other, the splitting position, which involves both PP and nucleoside portions. The latter is the one necessary for contraction, but it is still a matter for argument whether the adsorption itself (Morales *et al.* 1955) or the subsequent splitting (cf. Weber & Portzehl, 1954) is the primary cause of contraction. For relaxation, on the other hand, only the PP group is operative and, as we have seen, the nucleoside portion can be entirely absent.

As a complication to a simple explanation such as this, however, one should point out that not only is there an ion requirement for splitting, but Mg is also necessary for the full manifestation of the plasticizing effect (Mommaerts, 1948; Bendall, 1953).

The dual role of ATP in the interaction of actin and myosin is a fascinating invention of Nature, if, as seems likely, it is a homeostatic mechanism in which an enzyme reaction is inhibited by excess of its substrate. Even so, it does not yet seem possible to extrapolate the findings to the really crucial problem of how these reactions are linked to the molecular changes which constitute the very act of contraction, and this must be the main concern of muscle biochemistry in the future.

[2] I: ionic strength.—ED.

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INTERACTIONS OF ACTOMYOSIN AND ADENOSINETRIPHOSPHATE

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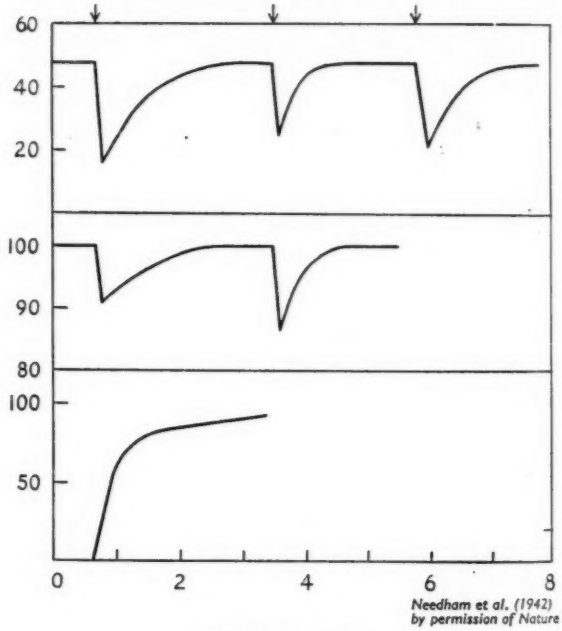
Up to 1939 much of the interest in muscle biochemistry was focused on two main aspects which at that time appeared unconnected, namely the metabolism of the phosphate esters in relation to glycolysis and the structural protein myosin. The demonstration that practically all the adenosinetriphosphatase activity of muscle was associated with the myosin fraction (Engelhardt & Ljubimowa, 1939) was the first indication of an interaction between adenosinetriphosphate (ATP) and myosin and was of special significance because it provided a vital link between metabolic processes and a structural component of the muscle cell.

This finding was rapidly confirmed (Needham, 1942; Bailey, 1942) and, despite many claims to the contrary, no satisfactory separation of the ATPase activity from myosin has yet been achieved. Working in Cambridge, the Needhams and collaborators (Needham, Kleinzeller, Miall, Dainty, Needham & Lawrence, 1942; Dainty, Kleinzeller, Lawrence, Miall, Needham, Needham & Shen, 1944) discovered that the addition of ATP produced a fall in the viscosity of myosin solutions, an effect which was reversible in so far that when the added ATP was hydrolysed the viscosity rose to its original value and the whole cycle could be repeated by the addition of fresh amounts of ATP (fig. 1).[1] Independently, in Hungary, at about the same time A. Szent-Györgyi and his collaborators had made similar observations and carried their investigations further (Banga & Szent-Györgyi, 1942; Szent-Györgyi, 1945). It was shown that, when minced muscle was extracted for a short period with salt solutions, myosin (myosin A) was the main structural protein passing into solution, but if extraction was prolonged another structural protein called actin (Straub, 1942, 1943) was also extracted. The Hungarian workers suggested that actin and myosin formed a complex known as actomyosin (myosin B) which gave highly viscous solutions, the viscosity of which was dramatically lowered on addition of ATP whereas that of pure myosin was unchanged. Consequently the effects obtained by the Needhams are explained on the basis that actomyosin was present in their myosin preparations.

Another important observation of the Hungarian workers, and one which is perhaps more directly comparable with the in-vivo contractile process, was made using precipitated threads of actomyosin. These threads, which were obtained by squirting the actomyosin solution through a fine orifice into a solution of low ionic strength (e.g. 0.05 M-KCl), shortened to $\frac{1}{3}$–$\frac{1}{4}$ of their original length on addition of very low concentrations of ATP ($\sim 1 \times 10^{-3}$ M). Unlike the contraction of muscle, however, the shortening of precipitated actomyosin threads was isodimensional; the protein concentration rose from 2–3% to 30–50% after the application of ATP and consequently was accompanied by a considerable squeezing out of water—a process known as synaeresis. Despite these and other differences from in-vivo contraction, it seemed likely from the studies described above that the fundamental contractile process involved the interaction of actin, myosin and ATP, and this system has since been widely studied from chemical, physical and physiological aspects.

FIG. 1. EFFECT OF ATP ON THE BIREFRINGENCE AND VISCOSITY OF ACTOMYOSIN SOLUTIONS



Needham et al. (1942) by permission of Nature

Abscissae: time (hr.)
Arrows mark the time of addition of ATP.

Top curve
Ordinates: flow-birefringence ($\Delta°$)

Middle curve
Ordinates: relative viscosity (η_i/η_0)

Bottom curve
Ordinates: PO_4 split off (%)

The figure illustrates the correlation between ATP hydrolysis and the return of viscosity and birefringence to their original values.

[1] See the paper by D. M. Needham, "Energy production in muscle", p. 194 of this number of the Bulletin, for other developments of this work.—ED.

1. Actomyosin Systems

The actomyosin used for such studies may be the so-called "synthetic" actomyosin prepared by mixing purified samples of actin and myosin in the same proportions as they occur in the cell (approximately 3 parts of myosin to 1 part of actin in rabbit skeletal muscle (Hasselbach & Schneider, 1951)), or "natural" actomyosin obtained by extracting the muscle under conditions such that both pass into solution. Although ATP produces a similar drop in the viscosities of "synthetic" and "natural" actomyosins, some authors claim that the molecular changes occurring in the two systems differ (cf. Morales, Botts, Blum & Hill, 1955). It is possible that, when "natural" actomyosin is prepared, the complex is extracted with the component proteins oriented with respect to one another as they are in situ, whereas simply mixing separately isolated samples of the two proteins produces a more randomly oriented system. Furthermore, natural actomyosin may contain additional components which are lost when the actin and myosin are purified separately.

Actomyosin is soluble at $I > 0.3$ and, as most physicochemical studies can only be made in solution, much of our knowledge of the effect of ATP on this system has been obtained at these higher salt concentrations.[2] Such studies are of great value in studying the nature of the interaction, but considerable differences in both physical and chemical behaviour exist when actomyosin in the precipitated (or gel form) is treated with ATP. The latter condition is more comparable to the state in which the complex exists in situ in the myofibril, and precipitated actomyosin ($I = 0.05$–0.15) forms a convenient model system for studying both contractile and enzymic behaviour. It does appear that once the actomyosin has been extracted, although qualitatively similar to the in-situ actomyosin complex, its contractile and enzymic behaviour shows certain quantitative differences. To some extent these can be ascribed to the fact that actomyosin gel precipitated under normal conditions rarely contains more than 2–3% protein and is composed of randomly oriented protein filaments, whereas the myofibril consists of 15–20% protein, most of which is actomyosin, laid down as filaments which are precisely oriented with respect to the axis of the myofibril. Preparations more suitable for studying the behaviour of actomyosin systems comparable to those in situ are provided, therefore, by isolated myofibrils (Schick & Hass, 1949; Perry, 1952, 1956b) freed from sarcoplasm and other cell components, and by glycerated fibre preparations (Szent-Györgyi, 1949). The latter are usually prepared by storing strips of rabbit psoas muscle held extended at resting length in 50% (v/v) aqueous glycerol for several days at −10° C. This process extracts much of the soluble sarcoplasmic protein and leaves the myofibrils intact, oriented as they were in vivo—a kind of contractile skeleton of the muscle cell.

2. ATP and the Other Nucleoside Phosphates of Muscle

ATP is the most abundant nucleoside triphosphate occurring in muscle, but small amounts of other triphosphates, namely inosinetriphosphate (ITP), uridinetriphosphate (UTP) and guanosinetriphosphate (GTP) are also present (Bergkvist & Deutsch, 1953). These other triphosphates are split by actomyosin and will cause similar physical changes in the system but, as might be expected, slight differences exist in the ionic conditions necessary to produce the changes and in the rate at which they occur. Although it cannot be stated with certainty, the evidence is strong that ATP itself is the important nucleoside triphosphate in the contractile process. This follows from the relatively large amount present in muscle and a comparison of the action of ATP with that of the other nucleoside phosphates on glycerated fibre-model systems (Weber, 1956). For this reason and the more compelling one that much less information is as yet available regarding the behaviour of the other nucleoside triphosphates, most attention will be devoted to the interaction effects of ATP. In the main, discussion will be concerned with the actomyosin system as found naturally in the myofibril.

As has been already indicated, the addition of ATP to the actomyosin system initiates enzymic and physical changes. The precise interrelation of these events is subject to controversy and in the first instance these effects of ATP will be discussed independently.

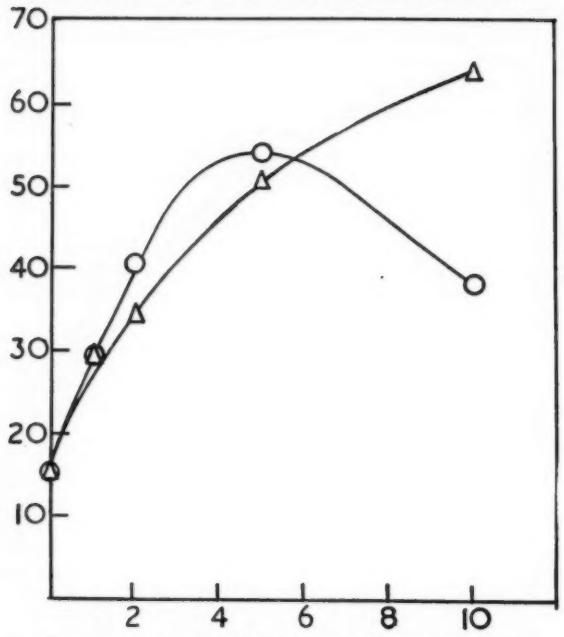
3. Enzymic Activity of Actomyosin

All actomyosin preparations possess adenosinetriphosphatase activity, catalysing the hydrolysis of ATP to adenosinediphosphate and *o*-phosphate. This activity is a property of the myosin component. Purified actin preparations have no ATPase activity, nevertheless the presence of this protein has a profound effect on some features of the enzymic activity of myosin. The ATPase activity of myosin itself has been widely studied, but caution must be exercised in relating the results of enzymic studies made on purified myosin to events in the muscle cell. For example, the ATPase of myosin is strongly activated by calcium, whereas magnesium inhibits the enzyme; indeed, when both cations are present, magnesium is strongly antagonistic to the activation produced by calcium. For this reason it has been considered (Mommaerts & Seraidarian, 1947; Bravermann & Morgulis, 1948) that in situ the myosin ATPase would be virtually inactive because the concentration of calcium and magnesium in muscle is such that the enzyme would be strongly inhibited. Such arguments ignore the fact that at low ionic strength actomyosin is activated by magnesium as well as by calcium and that these metals do not have the same antagonistic effect as with the myosin ATPase (see fig. 2)—in fact under certain conditions they have a pronounced synergistic action on the myofibrillar ATPase (see fig. 3). Precipitated "synthetic" and "natural" actomyosin gels are activated by magnesium at $I = 0.05$ but, as the salt concentration increases, the extent of this activation falls until at $I = 0.15$–0.20 the magnesium activation is absent. In contrast, the degree of activation achieved with calcium over this range of ionic strength may actually increase although at still higher salt concentrations it begins to fall off. Actomyosin in the naturally occurring gel form, i.e., intact myofibrils isolated from fresh or glycerated muscle, shows similar behaviour, and significantly the magnesium activation is much less sensitive to increasing ionic strength, i.e., magnesium still activates strongly in 0.16 M-KCl, which is considered to be isotonic with the muscle cell contents. It must be concluded therefore that magnesium can strongly activate the ATPase activity of the actomyosin in situ (myofibrillar ATPase).

The effect of salt on the magnesium activation of the actomyosin ATPase has been explained (Szent-Györgyi, 1945) as being due to dissociation into actin and myosin at higher ionic strength, so that enzymically the system behaves just like myosin itself, i.e., calcium activates whilst magnesium inhibits. Certainly at $I > 0.3$ the presence of F actin appears to have little effect on the enzymic activity of myosin.

[2] I: ionic strength.—ED.

FIG. 2. ACTIVATION OF THE ATPase OF MYOFIBRILS BY CALCIUM AND MAGNESIUM



Incubations carried out in 0.05 M-aminotrishydroxymethylmethane (Tris) buffer pH 7.4, and 5×10^{-3} M-ATP at 20° C.
Ionic strength ~ 0.06
Abscissae: concentration of activator (M $\times 10^{-3}$)
Ordinates: P liberated (μg./min./ml. suspension)
O——O $MgCl_2$
Δ——Δ $CaCl_2$

Calcium and magnesium have long been recognized to exert profound effects in biological systems. Their behaviour is often antagonistic and it is of great interest that they show marked differences in behaviour in the myofibrillar ATPase system. A feature of the magnesium-activated myofibrillar ATPase is that, at higher concentrations of ATP, enzymic activity is greatly inhibited (Hasselbach & Weber, 1953). It has been demonstrated recently with isolated myofibrils (Perry, 1956a, 1956b) that the level at which ATP becomes inhibitory is related to the concentration of magnesium in the system. The precise quantitative relation between the magnesium concentration and that of the ATP required to produce this effect depends on the ionic strength and the history of the myofibrils. In general, with myofibrils isolated from fresh or glycerated muscle and assayed in a medium of $I = \sim 0.16$ in the presence of 0.005 M-$MgCl_2$, inhibition begins to set in when the molar concentration of ATP exceeds that of the magnesium. From this observation it follows that, if most of the free magnesium present in the cell is in some way rendered unavailable to the myofibrillar ATPase system, then, at constant ATP level, ATPase activity will be at a low level (see section on relaxation). With calcium as the activating cation, increasing the ATP concentration over a similar range does not produce a marked inhibition. In fact, if calcium is present as well as magnesium, the substrate inhibition effect does not take place when the ATP concentration exceeds that of the magnesium. Calcium is strikingly effective in preventing this inhibition, for substrate inhibition does not occur if calcium is present in amounts as low as $\frac{1}{20}$ of the magnesium concentration. In fig. 3 are illustrated experiments showing the effect of calcium on inhibition of the magnesium-activated ATPase of isolated rabbit myofibrils in the presence of excess ATP.

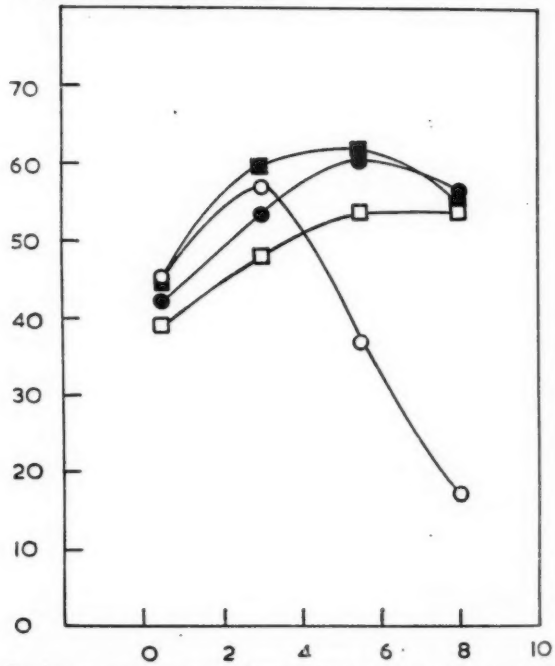
" Synthetic " actomyosin made from purified myosin and actin will split only the terminal phosphate from ATP, but " natural " actomyosin, myofibrils and glycerated fibres are usually contaminated with myokinase and 5-adenylic deaminase. There is no evidence that breakdown of ATP beyond the ADP stage is associated with contraction, but in the presence of these two enzymes ATP is converted by actomyosin systems to inosinic acid (see Perry, 1956c).

4. Physical Changes Induced by ATP in the Actomyosin System

a. Actomyosin Solutions

Both F actin and myosin exist in solution as long asymmetric molecules and if, in the absence of ATP, certain centres on the myosin molecule have an affinity for other specific centres on the actin, it follows that an anastomosed network of protein filaments would form and the system would

FIG. 3. EFFECT OF CALCIUM ON THE SUBSTRATE INHIBITION OF THE MAGNESIUM-ACTIVATED MYOFIBRILLAR ATPase



Conditions were similar to those of fig. 2, with 5×10^{-3} M-$MgCl_2$ present in all cases.
Abscissae: concentration of ATP (M $\times 10^{-3}$)
Ordinates: P liberated (μg./min./ml. suspension)
O——O 5×10^{-3} M-$MgCl_2$
■——■ 5×10^{-3} M-$MgCl_2$ and 5×10^{-4} M-$CaCl_2$
●——● 5×10^{-3} M-$MgCl_2$ and 2×10^{-3} M-$CaCl_2$
□——□ 5×10^{-3} M-$MgCl_2$ and 5×10^{-3} M-$CaCl_2$

possess a high viscosity. Such a structure has been observed by electron-microscope examination of actomyosin sols (Astbury, Perry, Reed & Spark, 1947), and the sedimentation constants measured in the ultracentrifuge indicate that the particles present have an extremely high particle weight (Weber & Portzehl, 1952). The orthodox interpretation of the viscosity drop obtained on addition of ATP to actomyosin sols ($I > 0.3$) has been that the triphosphate breaks the links which bind the actin to myosin so that these two proteins can now exist independently. Support for this view is presented by the fact that in the ultracentrifuge the myosin component can be recognized as sedimenting with its characteristic velocity (Weber & Portzehl, 1952). Furthermore the logarithm of the viscosity of the actomyosin in the presence of ATP can be expressed as the sum of the logarithm of the viscosity of the actin and myosin taken independently. The anastomosed structure which is observed on electron-microscope investigation of actomyosin disappears on addition of ATP. Such observations suggested dissociation into smaller units (Astbury *et al.* 1947), although some workers (Jordan & Oster, 1948) have considered electron-microscope and light-scattering studies to indicate evidence of a coiling of the particles. Other investigations involving light-scattering studies with "natural" actomyosin preparations have been interpreted to indicate that on addition of ATP the actomyosin micelle lengthens and then shortens again (Blum & Morales, 1953). Clearly inconsistencies exist in the description of the molecular changes induced by ATP in actomyosin sols. Morales *et al.* (1955) explain some of them on the basis that differences exist in the effect of ATP in "synthetic" and "natural" actomyosins, but until both types of actomyosin are studied with the same physical technique and are demonstrated to yield different results, it would be unwise to make this assumption.[3] Tsao (1953) using the polarization of fluorescence technique (Weber, 1952), which is of great value for studying molecular changes in solution, found that the state of polymerization of actin itself was changed by the addition of ATP to actomyosin (see article by Bailey, p. 183 of this number of the Bulletin).

Survey of the literature indicates that as yet we have no generally acceptable picture of the nature of the change produced by ATP on the actomyosin complex in solution. Several facts do emerge, however:

i. The change is a reversible one and the cycle can be repeated on addition of fresh ATP.

ii. Other nucleoside triphosphates—ITP, UTP, GTP, tripolyphosphate and inorganic pyrophosphate—will bring about a viscosity drop.

iii. Certain sulphydryl groups on the myosin molecule must remain intact for combination of actin and myosin, as is evidenced by a viscosity drop on addition of ATP (Bailey & Perry, 1947).

Undoubtedly the changes induced by ATP on actomyosin sols reflect important aspects of the interaction, but they do not closely parallel contraction. Perhaps they help best in understanding the physical differences which exist between resting and rigor mortis muscle. In the former, adequate ATP is present, consequently the constituent actin and myosin filaments of the myofibril are not interlinked and, as they can move independently, the muscle is freely extensible. ATP is absent, or present in very low concentration, in rigor mortis muscle and as a result the filaments of actin and myosin are linked at their points of interaction and the whole structure becomes rigid and inextensible—the relation between the actin and myosin corresponding to that occurring in the actomyosin sol.[4]

[3] See Blum, J. J. & Morales, M. F. (1956) *Fed. Proc.* **15**, 20, for a recent reinvestigation of this aspect.

b. Actomyosin Gels

Actomyosin gel, particularly in the form of isolated myofibrils and glycerated fibres, offers a satisfactory model system with which to study the contractile process. The evidence that the application of ATP to isolated myofibrils causes changes in structure identical to those observed in situ is now impressive (Ashley, Porter, Philpott & Hass, 1951; Perry & Horne, 1952; Hanson, 1952; Hanson & Huxley, 1955). Furthermore, when ATP is applied to glycerated fibre models, tensions of 0.5 kg./cm.^{2}–5.0 kg./cm.^{2} (Weber & Portzehl, 1954), which compare well with those exerted by living muscle, are obtained. Threads made from precipitated actomyosin exert much lower tensions, although some improvement can be obtained if the protein concentration of such threads is increased and the micelles are oriented in the direction of the fibre axis (Portzehl & Weber, 1950; Portzehl, 1951).

When unloaded or lightly loaded actomyosin threads and glycerated fibres are treated with ATP, shortening down to 25–30% of the original length occurs, whereas in-situ shortening to less than 65% of resting length is rare. The extensive shortening of model systems can be prevented by adequate loading but, under certain conditions, living muscle can shorten as extensively as the models, e.g., when muscle shortens to the delta state (Ramsey, 1947; Ramsey & Street, 1940).

An early observation made with actomyosin threads which is of special significance was that if such threads (of low protein content) were loaded and treated with ATP an increase in length was obtained (Engelhardt, 1946; Buchtal, Deutsch, Knappeis & Münch-Petersen, 1947). This means that the tensile strength of the thread has fallen and suggests that, in addition to its role as a contracting agent, ATP can also exert a plasticizing action. With threads of higher protein concentration, and of course with glycerated fibres which are much more capable of transmitting the tension developed by the shortening of the individual micelles, the whole thread responds by contraction on addition of ATP. This dual function of ATP, as a plasticizing and as a contracting agent, is of special significance in understanding the complete cycle of muscle action, which involves both contraction and relaxation.

5. Enzymic Activity and Contraction

The contracting action of ATP and the other nucleoside triphosphates is closely related to the enzymic function of the actomyosin, but the precise nature of this relation is still a matter for controversy. In the older view it is considered that dephosphorylation of ATP must take place for contraction to occur; in other words, even if ATP is present, contraction will not take place unless phosphate is removed from the ATP. Evidence supporting this hypothesis has been provided by comparing the rate of shortening of muscle models with the rate of ATP dephosphorylation (Weber & Portzehl, 1952, 1954; Bendall, 1953a; Bozler & Prince, 1953). Comparison of the relative efficiency in inducing the shortening of isolated myofibrils of ATP maintained fully phosphorylated by the creatine phosphokinase system with a similar concentration of the nucleotide alone, also supports this view (Perry, 1954). Although the evidence is impressive there are cases where the

[4] See also Bate-Smith & Bendall, p. 230 of this number of the Bulletin.—Ed.

correlation between extent of shortening and the rate of ATP dephosphorylation is not so close. For example, Bowen & Kerwin (1955) have presented data which show that changes in the ionic conditions reduce the rate of dephosphorylation of ATP, yet at the same time accelerate the shortening of glycerated fibres. Likewise, Morales *et al.* (1955) consider that further evidence that dephosphorylation and contraction are not necessarily associated is provided by the fact that tripolyphosphate is dephosphorylated by actomyosin, although this polyphosphate has very weak powers of inducing contraction (Friess & Morales, 1955). Such results and others have been used to support the view that the binding of ATP to the contractile system is the event that induces contraction (see Morales *et al.* 1955) and that dephosphorylation occurs after contraction takes place. Irrespective of the merit of the latter hypothesis, it is not altogether satisfactory to use evidence in support of it based upon the lack of correlation between splitting of ATP and contraction. Contraction of models involving an observable change occurs over a much narrower range of ionic conditions than does the hydrolysis of ATP.

A decisive demonstration of the phosphorylation of myosin during the hydrolysis of ATP would be valuable in deciding between these theories. Using ^{32}P, Koshland, Budenstein & Kowalsky (1954) were unable to provide evidence of such a process, although recently Weber (1956) has reported that phosphorylation of myosin can be demonstrated but only in the presence of an inhibitor, Fouadin, which prevents the liberation of phosphate from ATP.

Physiological evidence, although not conclusive, suggests that contraction is the active process (Hill, 1949). If dephosphorylation accompanies contraction this must be so, but on the other hand contraction could also be the active process if it is mediated by the binding of ATP. Pertinent from this point of view are the results of Oullet, Laidler & Morales (1952) which suggest that a large part of the free energy change occurring during ATP hydrolysis is associated with the process of binding ATP to actomyosin.

6. Relaxation

In relaxed muscle the myofibril is maintained at the resting length and, so far as can be judged by biochemical analysis, inorganic phosphate is liberated at a low rate. In ionic media similar to that of the cell, isolated actomyosin systems—whether as threads, myofibrils or glycerated fibres—contract violently on addition of ATP at the concentration present in muscle, and obviously in situ this reaction is inhibited until the muscle cell is stimulated. The present view is that there exists in resting muscle some relaxing factor or factors which prevent contraction by inhibiting the ATPase activity of the myofibril. In effect the relaxing factor modifies the interaction of ATP with the myosin system.

The first indication that muscle contained a factor which inhibited the ATPase activity of the myofibril came from the investigations of Bailey & Marsh (1952) and Marsh (1952). Marsh noted also that this factor was present in sarcoplasmic extracts and found that in the presence of ATP it produced a lengthening of muscle fibre fragments. He considered that the same factor was responsible for both effects and played an important role in relaxation in living muscle. Bendall (1953a) showed that the Marsh factor would bring about relaxation in glycerated fibres previously contracted with ATP (fig. 4).

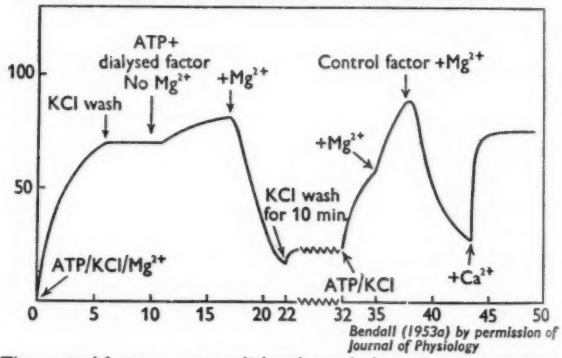
Working independently in the USA, Bozler (1951) was the first to demonstrate decisively that a complete contraction-relaxation cycle could be obtained with the glycerated fibre preparation. He found that the applications of low concentrations of ATP to glycerated fibre preparations, which had been stored for only a few days and very lightly washed, produced contraction; whereas, when higher ATP concentrations were used, a slight contraction was followed by relaxation. On exhaustive washing, the relaxing effect was not obtained, but ATP produced contraction only, and it was apparent that Bozler's preparations contained some relaxing factor which was removed on washing the fibres.

It is clear that the factor present in lightly washed glycerated fibres is identical with that discovered by Marsh and later shown to be effective in the well-washed glycerated fibre system by Bendall. More recently Bendall (1954) has considered this factor to be identical with myokinase, but Japanese workers (Kumagai, Ebashi & Takeda, 1955) have produced evidence which indicates that two sarcoplasmic factors are concerned, neither of which is necessarily myokinase.

Apart from these effects with apparently physiological relaxing factors, Weber and collaborators (see Weber & Portzehl, 1954) were able to obtain relaxation in fibre models by employing conditions in which the ATP-splitting action of actomyosin is inhibited. This was achieved by allowing a fibre model to shorten in the presence of ATP, and then at the height of contraction adding a sulphydryl poison, Salyrgan, which destroyed the ATPase activity. So long as ATP was present, the poisoned fibre model relaxed. Under such conditions the plasticizing action of the ATP allows the actomyosin to relax, as the enzymic activity which induces contraction is no longer active. There is some evidence that this plasticizing action of ATP, which is responsible for relaxation in actomyosin gels and the viscosity drop obtained with sols, is associated with the polyphosphate grouping; for relaxing action has also been obtained with inorganic polyphosphates, in particular with pyrophosphate (Weber, 1951; Bozler, 1951; Bendall, 1953b) which is not split by myosin ATPase.

Certain features, however, are common to all the relaxation

FIG. 4. EFFECT OF DIALYSED RELAXING FACTOR ON THE ATP-INDUCED SHORTENING OF GLYCERATED FIBRES IN THE ABSENCE AND PRESENCE OF MAGNESIUM AND CALCIUM



Bendall (1953a) by permission of Journal of Physiology

The control factor was an undialysed sample from the same stock as the dialysed.

Concentrations: 6×10^{-3} M-ATP; 0.16 M-KCl; 4×10^{-3} M-$MgCl_2$; 2×10^{-4} M-$CaCl_2$

Abscissae: time (min.)

Ordinates: shortening or work done (percentage of maximum)

systems which have been described. They are the following:

i. ATPase activity is low;

ii. a plasticizer must be present; this may be ATP itself or a polyphosphate;

iii. magnesium appears to be essential.

As indicated earlier, study of the effects of various relaxing factors on the ATPase activity of isolated myofibrils (Perry, 1956b) has suggested that the relation between the concentrations of available magnesium and ATP is very critical in deciding the level of ATPase activity. In general, when the free magnesium level is relatively low the ATPase is much inhibited, and this condition is characteristic of that found in relaxed fibre models. It is considered (Perry, 1956b) that relaxing factors act in one of the following ways:

i. by reducing the effective magnesium concentration—for example, inorganic pyrophosphate (Bendall, 1953b) has long been known to complex with magnesium (Rogers & Reynolds, 1949). The Marsh factor may act in this way also;

ii. by increasing or maintaining the ATP concentration at a high level; for example, this is probably the explanation of the relaxing action of creatine phosphokinase (Goodall & Szent-Györgyi, 1953; Lorand, 1953; Bozler, 1954).

Good evidence that the condition of substrate inhibition occurs during relaxation in models is provided by the fact that calcium inhibits the relaxing factor (Bendall, 1953a, fig. 4). Enzyme experiments with isolated myofibrils have clearly indicated that higher concentrations of ATP do not inhibit the magnesium-activated ATPase if quite low concentrations of calcium are present (see fig. 3). The results of these in-vitro experiments with fibre models and isolated myofibrils can be used with some justification to speculate upon the conditions within the living muscle. It is suggested that the low rate of ATP hydrolysis by the myofibril in resting muscle is due to the fact that most of the magnesium present is bound to the relaxing factor so that, relative to the available magnesium, ATP is in considerable excess. This means that contracting activity is low, but the ATP is fully active as a plasticizing agent for the actomyosin system, and the muscle is readily stretched. Addition of small amounts of calcium to models in a similar state strongly stimulate the ATPase activity, and it is possible to explain the contraction obtained by injecting calcium through a micropipette into the isolated resting single fibre (Heilbrunn & Wiercinski, 1947; Niedergerke, 1955) as due to this effect. The contraction which accompanies stimulation must mean a sharp increase in ATPase activity. How this is achieved is a matter for further speculation, although from the enzymic properties of the myofibril two possibilities suggest themselves: removal of the substrate inhibition by liberation of the magnesium bound by the relaxing factor or, alternatively, momentary liberation of a lower concentration of free calcium. Whatever the mechanism, it seems clear that in living muscle ATP alternates between acting on the actomyosin system as a contracting agent which results in a high rate of splitting, and acting as a plasticizing agent with the low rate of splitting characteristic of relaxation. To relate this change of actomyosin state with the electrical changes occurring at the sarcolemma on stimulation is one of the great problems of muscle physiology.

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ENERGY PRODUCTION IN MUSCLE

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1 Lactic acid and contraction
2 Creatine phosphate and contraction
3 Adenosinetriphosphate and contraction
4 Oxidative re-synthesis of adenosinetriphosphate
5 Attempts at investigation of the primary reaction
References

1. Lactic Acid and Contraction

There is no doubt that the energy used by muscle in carrying out its functions of performing work and maintaining tension is ultimately derived from chemical reactions going on within it. During the latter half of the nineteenth century much experimental effort and much controversy were lavished on the question of the nature of these reactions; but it was not until the work of Fletcher & Hopkins (1907) that quantitative and reproducible results were obtained. This advance was due to their care in reducing to a minimum any stimulation of the muscle during the process of fixation. They were thus able to show definitely, for the first time, that fatigue and rigor are always accompanied by lactic acid production. Later, Parnas & Wagner (1914) were the first to demonstrate that the source of the lactic acid is the muscle glycogen. Now it was known from the difference in the heats of combustion of glycogen and lactic acid that the production of lactic acid from glycogen should go on with heat output (16,200 cal./g. mol. lactic acid formed according to Meier & Meyerhof, 1924). It was assumed —and justifiably as we shall see—that here was a reaction which could provide energy for contraction. This view was upheld by the results of Meyerhof's extensive studies showing the proportionality during anaerobic contraction between lactic acid formed and tension production or work done (see Meyerhof, 1920, 1921).

Early experiments on the heat production during muscle contraction and relaxation (the initial heat) showed that its magnitude (Peters, 1913; Weizäcker, 1914), its time relations (Hill & Hartree, 1920) and its relation to tension production (Hill, 1928b) are all of exactly the same value whether the muscle contracts in nitrogen or in oxygen. This clearly indicates that the chemical reactions underlying contraction are non-oxidative; later D. K. Hill (1940a) showed that increased oxygen consumption does indeed begin only after activity is over. If the conditions are anaerobic, the period after activity shows only small and variable heat exchange, the heat production; spread over about thirty minutes, being less than 20% of the initial heat (see D. K. Hill, 1940b, for the latest experiments and references to earlier work). When oxygen is present there is large production of heat during recovery, almost equal to the initial heat (Hill, 1928b).

2. Creatine Phosphate and Contraction

It was another twenty years before doubt was cast on the theory of carbohydrate breakdown to lactic acid as the only energy-providing reaction. But then Lundsgaard (1930a) showed contraction without lactic acid formation in muscle poisoned with iodoacetate, and found that in such cases tension production was proportional to breakdown of creatine phosphate. The latter substance (phosphagen) had been discovered in muscle by Eggleton & Eggleton (1927a, 1927b) in Britain and independently by Fiske & Subbarow (who were the first to make its structure clear) (1927, 1929a) in the United States of America. Eggleton and Eggleton had found phosphagen to decrease in amount in the muscle during contraction, with re-synthesis on oxidative recovery. Nachmansohn (1928) in Meyerhof's laboratory showed that even in nitrogen in the 30 seconds immediately after relaxation there was re-synthesis of about 30% of the creatine phosphate.

Nachmansohn (1928, 1929) compared creatine phosphate breakdown with tension development, and found that in a series of contractions the breakdown is much greater during the early contractions than the later ones. This behaviour of creatine phosphate was puzzling from the point of view of the lactic acid theory of contraction. First, experience up to that time had shown proportionality between tension production, heat liberation and lactic acid formation (see Hill, 1928a): but if the early stages of tension production were characterized by creatine phosphate hydrolysis, known to be an exothermic reaction, why was there not greater heat production at this time? Secondly, how was the rapid anaerobic re-synthesis of creatine phosphate following contraction to be explained?—i.e., the occurrence of an endothermic reaction during a short interval of time when heat exchange was very small and certainly no equivalent heat absorption could be detected (Hartree & Hill, 1928). Indeed, the suggestion was made at this time that no breakdown happened in vivo, but rather an "unstabilization" of the creatine phosphate, rendering it liable to be broken down by the chemical treatment of estimation. (For discussion of this point of view see Hill, 1928a; Hartree & Hill, 1928; Nachmansohn, 1928; Meyerhof, McCullagh & Schulz, 1930.)

The solution of this dilemma began with the observation of Lipmann & Meyerhof (1930) that the first few of a series of short tetani led to a change towards the alkaline side in the muscle. This was connected by Lipmann and Meyerhof with the earlier observation (Meyerhof & Lohmann, 1928c) that hydrolysis of creatine phosphate in vitro at physiological pH into free creatine and inorganic phosphate is accompanied by liberation of alkali. Thus it was shown that creatine phosphate breakdown must indeed be reckoned with in the contracting muscle itself, and the way was paved for the results of Lundsgaard.

Lundsgaard (1930a, 1930b) suggested that, in muscle poisoned with iodoacetate (which prevents lactic acid formation), the energy for contraction comes from creatine phosphate breakdown; he went further and suggested that even in normal muscle this might be true also, the role of carbohydrate breakdown being to supply energy for re-synthesis of creatine phosphate. Investigation of the very early stages of fatigue showed him that, with a lightly loaded muscle or with a short period of stimulation, lactic acid formation was less in proportion to the tension than under conditions where total tension production was greater; so we have the explanation of the constancy of the heat:tension ratio, although the

chemical reactions underlying the heat production vary with time in a contraction series (Lundsgaard, 1931).

Finally Lehnartz (1931) brought convincing evidence in support of the earlier claims of the Embden school that much of the lactic acid production is post contraction; this was confirmed by Meyerhof (1931) who showed that, with a 5-second tetanus at 3° C., as much as two-thirds may take place after relaxation. Here then we have the energy source of the anaerobic creatine phosphate re-synthesis. The smallness of the heat exchange (D. K. Hill, 1940b) indicates how efficient the process must be.

It should be mentioned that other phosphagens besides creatine phosphate occur, all being compounds containing the guanidino grouping. The bases concerned are shown below, and in each case the phosphate group replaces one hydrogen of the terminal amino group.

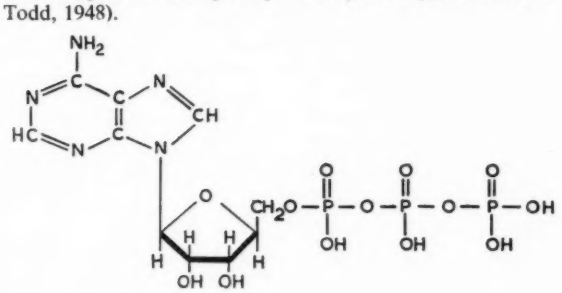
Creatine	$H_2N.C(:NH).N(CH_3).CH_2.COOH$
Arginine	$H_2N.C(:NH).NH.(CH_2)_3.CH(NH_2).COOH$
Glycocyamine	$H_2N.C(:NH).NH.CH_2.COOH$
Taurocyamine	$H_2N.C(:NH).NH.CH_2.CH_2.SO_3H$
Guanidylethylserylphosphate	$H_2N.C(:NH).NH.(CH_2)_2.O.(O)P(OH).O.CH_2.CH(NH_2).COOH$

Creatine phosphate is found in all vertebrate muscle (Eggleton & Eggleton, 1928) and in some invertebrate (Needham, Needham, Baldwin & Yudkin, 1932; Baldwin & Yudkin, 1950); arginine phosphate is characteristic of most invertebrate muscle examined (Meyerhof & Lohmann, 1928a, 1928b), but recently it has been shown that certain annelids contain, besides creatine phosphate, two new phosphagens—glycocyamine phosphate and taurocyamine phosphate (Thoai, Roche, Robin & Thiem, 1953; Hobson & Rees, 1955). Hobson (1955) has shown that phosphokinases are present which can phosphorylate the two bases at the expense of adenosinetriphosphate (ATP) (compare reaction (4) below).

Thoai & Robin (1954) have isolated guanidylethylserylphosphate from the earthworm, and shown the presence in its muscle of the corresponding phosphagen.

3. Adenosinetriphosphate and Contraction

The facts described above could bear the interpretation which Lundsgaard put upon them, but another interpretation is possible and seems to be the correct one—that both carbohydrate and creatine phosphate breakdown are supplying energy for the building up of a third substance, adenosinetriphosphate (ATP), hydrolysis of which yields energy (see below). This substance was discovered in muscle independently by Lohmann (1929, 1931) and by Fiske & Subbarow (1929b). Lohmann assigned to it the structure shown (1932, 1935) and this has been supported by Lythgoe & Todd (1945) and finally confirmed by its synthesis (Baddiley, Michelson & Todd, 1948).



The realization of the importance of ATP came directly out of the work of Lohmann (1934) on hydrolysis of creatine phosphate in cell-free muscle extracts. He found that, if such extracts are dialysed, they are incapable of hydrolysing creatine phosphate; the hydrolysis goes on only in the presence of adenylic compounds and could be shown to have two stages:

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{creatine phosphokinase}} \text{creatine} + \text{ATP} \quad (1)$$

$$\text{ATP} \xrightarrow{\text{ATPase}} \text{ADP} + H_3PO_4 \quad (2)$$

There were two most important consequences from this work. First, Lohmann deduced that ATP breakdown must precede creatine phosphate breakdown and thus becomes the energy-yielding reaction closest to contraction. Secondly, the realization dawned that, in reaction (1) (" Lohmann's reaction "), transfer of phosphate from one molecule to another, without intermediate formation of inorganic phosphate, is accompanied by transfer of free energy—a mechanism which has turned out to be of enormous biological importance.

If this then was the role of creatine phosphate, what could be said of the process whereby carbohydrate breakdown provided energy and phosphate for re-phosphorylation perhaps of creatine, perhaps of adenosinediphosphate (ADP)?

It was known that two stages in the breakdown of glycogen went on with output of heat, and this was taken as a sign that free energy might be available at these stages—the dephosphorylation of phosphopyruvate and the oxidoreduction between glyceraldehydephosphate and pyruvate. Experiments with muscle extract (Ostern, Baranowski & Reis, 1935; Needham & van Heyningen, 1935; Meyerhof & Lehmann, 1935) showed that phosphopyruvate, like creatine phosphate, reacts with adenylic compounds and is not dephosphorylated in their absence.

$$\text{Phosphopyruvate} + \text{ADP} \xrightarrow{\text{pyruvic phosphokinase}} \text{pyruvate} + \text{ATP} \quad (3)$$

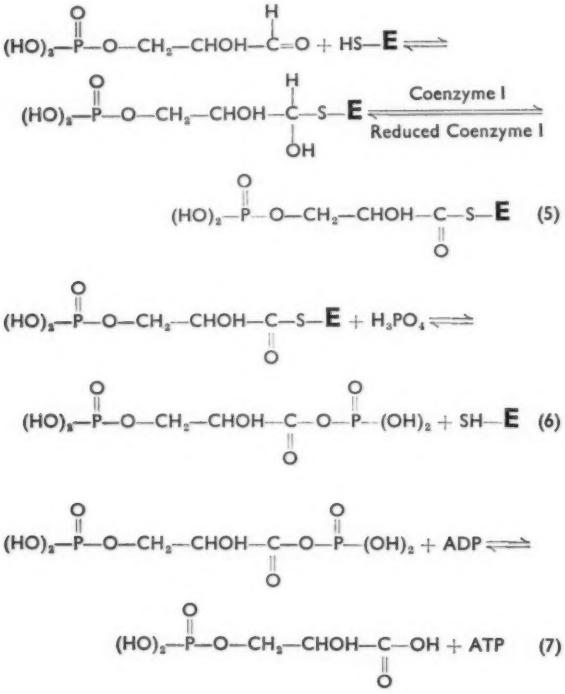
$$\text{ATP} \longrightarrow \text{ADP} + H_3PO_4$$

If creatine was present together with a catalytic amount of ADP, creatine phosphate was synthesized so that reaction (3) must be followed by

$$\text{ATP} + \text{creatine} \longrightarrow \text{ADP} + \text{creatine phosphate} \quad (4)$$

This is the reverse of reaction (1), and in fact direct demonstration of the reversibility of the Lohmann reaction has been made (Lehmann, 1936). With regard to the oxidoreduction, here again it was shown (Needham & Pillai, 1937; Meyerhof, Schulz & Schuster, 1937) that a synthesis of ATP can go on, one molecule of inorganic phosphate being esterified for every molecule of glyceraldehydephosphate oxidized. Warburg and his collaborators (Warburg & Christian, 1939; Negelein & Brömel, 1939) showed 1:3-diphosphoglyceric acid to be the phosphate donator here, and the sequence of reactions has recently been elucidated by the results of several different groups of workers (for references see Anfinsen & Keilley,

1954). The probable course of events is shown schematically below (reactions 5, 6, 7)—the reaction of the aldehyde group of the substrate with SH on the enzyme (**E**), triosephosphate dehydrogenase; the oxidation of this hemimercaptal; and then phosphorolysis of the enzyme-acyl compound (Racker & Krimsky, 1952; Segal & Boyer, 1953) to form the acyl phosphate.



In the years between 1932 and 1938 Meyerhof and his colleagues (Meyerhof & Lohmann, 1932; Meyerhof & Schulz, 1935; Meyerhof, Ohlmeyer & Möhle, 1938) studied the heats of hydrolysis of creatine phosphate, ATP, phosphopyruvic acid and other intermediate esters in glycogen breakdown. They came to the general conclusion that, for each g.mol. of phosphate set free, the heat of the reaction was about 12,000 cal. in the three cases mentioned; they also calculated the heat liberation on oxidation of glyceraldehydephosphate by co-enzyme I to be of the same value. These studies led to the early important distinction between compounds containing the guanidinophosphate, pyrophosphate or enolphosphate linkage on the one hand, and those containing the ordinary phosphate ester linkage on the other. The hydrolysis of the last sets free only about 3,000 cal. It was realized at this time that the important aspect of these reactions is the free energy change; but, since no method of measuring this had been devised, the heat of reaction was taken as a guide. Lipmann (1941), in a survey both illuminating and influential, coined the terms "energy-rich" and "energy-poor" phosphate bonds and began the long series of efforts, which still continue, to find by calculation and study of equilibria true values for the free energy of hydrolysis of these compounds (see Oesper, 1951).

The results described indicate the sequence of events in energy provision for contraction—firstly, dephosphorylation of ATP which, though not discernible in moderate contraction, is usually considered to be the essential reaction; secondly, immediate re-phosphorylation of ADP by reaction with creatine phosphate; later, re-phosphorylation of ADP by phosphopyruvate and diphosphoglycerate formed in carbohydrate breakdown.

4. Oxidative Re-Synthesis of Adenosinetriphosphate

The study of processes of oxidative phosphorylation has lagged behind that of anaerobic methods of ATP restitution; this is because the enzyme systems concerned are not water-soluble but are contained in cell particles, the mitochondria, which very readily suffer damage on separation from the cell. During recent years, however, much progress has been made in the study of these particles, especially of those from the liver. It has been shown that the ratio of phosphate esterified to oxygen consumed, the P:O ratio, may be as high as three or four. Since six oxygen molecules are concerned in the oxidation of one glucose molecule, some 36–48 energy-rich phosphate bonds may be expected. This is to be compared with the two energy-rich phosphate bonds made available on formation of lactic acid from one molecule of glucose. There is no doubt that, under normal physiological conditions, the efficient oxidative re-synthesis of ATP is much more important than the anaerobic re-synthesis; but in special circumstances the latter must often come into play. Oxidative phosphorylation in mitochondria of skeletal muscle (pigeon breast) has been studied by Chappell & Perry (1953) and in those of heart muscle by Slater (1955). Plate III, fig. A, illustrates the close contact between myofibrils and mitochondria.

It is interesting to remember that the first consideration of the possible mechanism of ATP synthesis during oxidative phosphorylation arose out of work on muscle. In the studies of Belitzer & Tsibakova (1939) on the quantitative relation of P esterified to O_2 consumed during oxidation of various substrates by minced muscle, it was observed that the P:O ratio was about 2; there was little doubt that this should be corrected to a higher value, owing to the ATPase activity of the mince. Belitzer and Tsibakova pointed out that the dehydrogenation of substrate could account for uptake of only one P atom per atom of O, and postulated that further P uptake was associated with the later stages of hydrogen transport to molecular oxygen. Thermodynamic considerations show that the free energy change during this passage is large enough to account for three or four phosphorylations. Thus from the charts given by Dixon (1948) the values of ΔF° per two hydrogen gram atoms at pH 7 may be gauged as approximately 12,000 cal. between the coenzyme I stage and the flavoprotein stage; 14,000 cal. between the latter and the cytochrome system; and 24,000 cal. between this last system and oxygen at one atmosphere pressure.

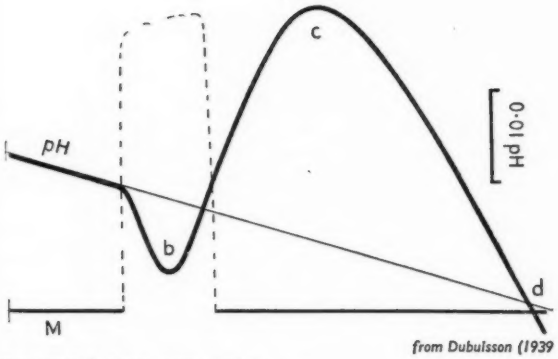
Although subsequent work on the mechanism of ATP synthesis during respiration has been done on other tissues, some results may be briefly mentioned here, since the same mechanisms no doubt operate in skeletal muscle. For the reactions concerned in the formation of energy-rich phosphate bonds during the dehydrogenation of α-oxoglutarate, see Kaufman (1955). This is the only case in the respiratory tricarboxylic acid cycle where phosphorylation at substrate level has been thoroughly investigated. Evidence for P

esterification in liver mitochondria during the passage of electrons from reduced coenzyme I to O_2 is given by Lehninger (1951); during oxidation of ferrocytochrome C with O_2, by Judah (1951) and by Nielsen & Lehninger (1954); and during reduction of ferricytochrome C, by Cooper, Devlin & Lehninger (1955) and by Slater (1955). The last-named used heart muscle mitochondria. Possible mechanisms for the P esterification have been considered by Hunter (1951); Slater (1953); Boyer, Falcone & Harrison (1954); Lehninger (1955) and Chance, Williams, Holmes & Higgins (1955).

5. Attempts at Investigation of the Primary Reaction

From all that is known of the interactions of ATP with actin and myosin (the proteins of which the myofibril is mainly composed), especially when we remember the ATPase activity of myosin, the conclusion seems inescapable that ATP breakdown is closely connected with contraction (see the article by Perry in this number of the Bulletin, p. 188). It is clear that direct evidence for this breakdown during normal contraction is desirable. In this connexion it is worth while to return for a moment to pH changes during contraction. The early experiments already mentioned (Lipmann & Meyerhof, 1930) used manometric methods which had a lag period of some 20 minutes. In the later experiments of Dubuisson (1939), a glass electrode was used in close contact with the muscle; the time lag was 2–3 seconds. In fig. 1, for a 4-second tetanus of frog gastrocnemius, a series of three pH changes are observed: (i) towards the acid side during the contraction (see b on fig. 1); (ii) towards the alkaline side (see c on fig. 1); (iii) towards the acid side (see d on fig. 1), the two latter being mainly post contraction. Examination of the effects of varying the initial pH of the muscle and of treatment with iodoacetate supported the view that (i) may be identified with ATP breakdown, (ii) with creatine phosphate breakdown and (iii) with lactic acid formation.

FIG. 1. CHANGES IN pH DURING 3–4 SECONDS' TETANUS OF FROG GASTROCNEMIUS



from Dubuisson (1939

Top record: 2-sec. time marker

Continuous thick line: record of pH changes accompanying muscle contraction. Downward deflection means fall of pH; pH calibration on right

b, c, d: phases i, ii, iii (see text)

Continuous thin line: presumed pH change in absence of contraction. M: base line of muscle response; dotted line: response of muscle to excitation

It was natural that, with the extensive use of labelled P compounds, the hope should arise that it might be possible to show increased turnover of ATP during contraction and in this way support the view that its breakdown is necessary for contraction. This hope has not been fulfilled, the great difficulty being the slowness of penetration of the injected inorganic ^{32}P from the blood stream into the fibres. The ^{32}P in the space outside the fibres will therefore remain for a long time of much higher isotopic concentration than the inorganic P inside the fibres, and so far it has not proved possible accurately to assess the labelled intracellular inorganic P. It has been shown (Kalckar, Dehlinger & Mehler, 1944) that calculations depending on assessments of the interfibrillar space are inadequate; and Ennor & Rosenberg (1952) in turn have shown that the perfusion technique proposed by Kalckar and his co-workers for removing the interfibrillar P before extracting the tissue for analysis gives no true picture. So far no satisfactory way of following the turnover of ATP within the fibres has been devised.

We come now to the recent attempts, making use of the new possibilities of micro-estimation by chromatographic and enzymic assay and the most refined techniques for rapid fixation, to show ATP breakdown at a time so soon after stimulation that re-phosphorylation of ADP would not yet have taken place.

Munch-Petersen (1953), using tortoise muscle at 20° C., found an increase of 7% in ADP on a single twitch; and Lange (1955) found both creatine phosphate breakdown and ADP formation at 0° C. upon contracture caused by immersion for 30 seconds in KCl solution, or 1–10 seconds in acetylcholine solution. Other investigators, however, have not succeeded in observing these phenomena under their own experimental conditions and it is striking that cases have been described where measurable performance of work took place without either ADP formation or creatine phosphate decrease. Fleckenstein, Janke, Lechner & Bauer (1954), using the rectus abdominis of the frog, were the first to observe that, at 0° C. after 1–2 seconds' tetanus stopped during the rise of tension, there was no change in ATP, ADP or creatine phosphate content; at 20° C., the creatine phosphate content was lowered after contraction but there was no change in ATP or ADP. These experiments were confirmed in later work (Fleckenstein, Janke, Davies & Krebs, 1954). The further interesting observation was made that, in the absence of ATP and creatine phosphate breakdown, an increase in inorganic P could be detected and an increase in an unknown compound, " fraction F ". The increase in inorganic P was enough to account for the work done, on the assumption that it was derived from an " energy-rich " phosphate. Creatine phosphate breakdown was shown to occur for some seconds after stimulation, suggesting that some form of re-phosphorylation was needed. The authors consider that the possibility of oxidative re-phosphorylation of ADP is ruled out.

Results bearing out this possibility of contraction at 0° C. without ATP breakdown are also reported by Mommaerts (1954, 1955). He used turtle muscle, stimulated electrically to a single twitch under anaerobic conditions and fixed during the rise of tension. He found no ADP or free creatine formation; he also considered that no possibility of

re-phosphorylation existed through carbohydrate breakdown or by means of any traces of phosphopyruvate in the tissue.

Two possible explanations of these results may be suggested: that ATP, having broken down, is at once re-phosphorylated by some unknown substance; or that some other compound than ATP can, at any rate under certain conditions, supply by its breakdown free energy directly to the myofibrils. H. H. Weber (1956) has given reasons for believing that none of the other nucleoside triphosphates known to occur in tissues could be this latter compound. Thus, although under the right conditions triphosphates of inosine, uridine or guanosine can cause tension to develop in fibre models, the maximum tension is only 50–70% of that with ATP; and if radioactive ATP and unlabelled ITP + IDP are used all together with a fibre model, the ITP-IDP concentration remains unchanged until all the ATP has been dephosphorylated and the inosine compounds do not become labelled. Still another possibility might be considered. It has often been postulated that transfer of the free energy available on ATP breakdown might involve actual phosphorylation of some group on the myosin (see, for example, Needham, Shen, Needham & Lawrence, 1941). It seems possible that, especially at low temperature, some phosphorylated protein formed as the result of a previous stimulation might persist in quantity sufficient to provide for a short contraction.

* * *

Thus we have seen that, during the first half of the present century, a whole succession of compounds has been brought forward, one after another fitting into its place in the complex cycle of processes providing energy for the muscular machinery. Each time the fundamental point of linkage between the chemical reactions and the physical configuration change has receded one step backward, eluding its pursuers. But this process seems now to be coming to an end, and we are perhaps within sight of that primary connexion, understanding of which will bring the solution of a problem sought by many investigators for more than fifty years.

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ENERGY PRODUCTION IN MUSCLE

(FIG. A)

D. M. Needham

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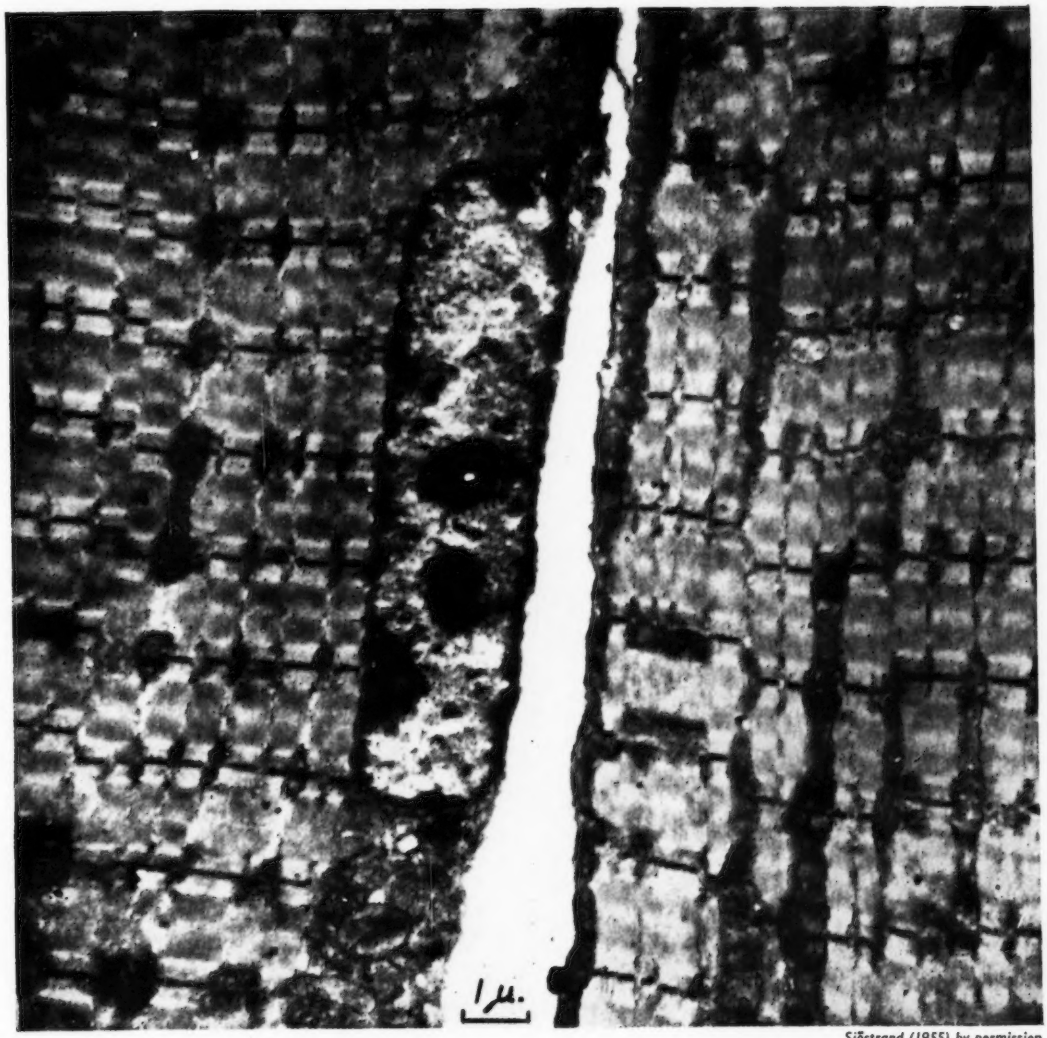

Sjöstrand (1955) by permission of Science Tools

FIG. A. Electron micrograph of an ultra-thin longitudinal section of skeletal muscle fibres, showing the striated myofibrils with mitochondria between them, and a nucleus at the edge of one fibre (magnification × 9,000)

COMPARATIVE PHYSIOLOGY OF MUSCLE

(FIGS. A—D)

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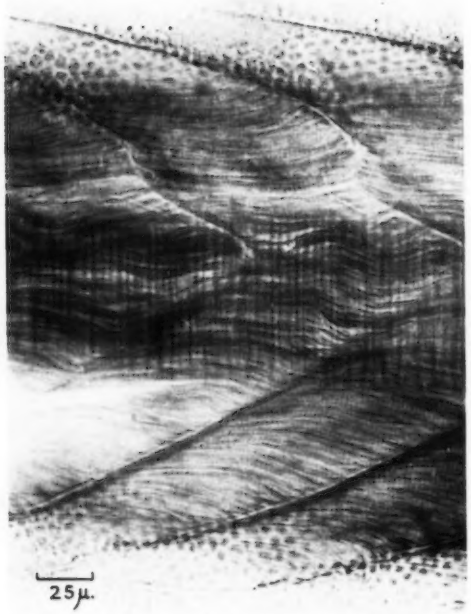

FIG. A. Whole mount of *Amphioxus* (head to right), near tail region. Note oblique insertion of muscle fibres on to V-shaped septa. The notochord can be seen through the tissue

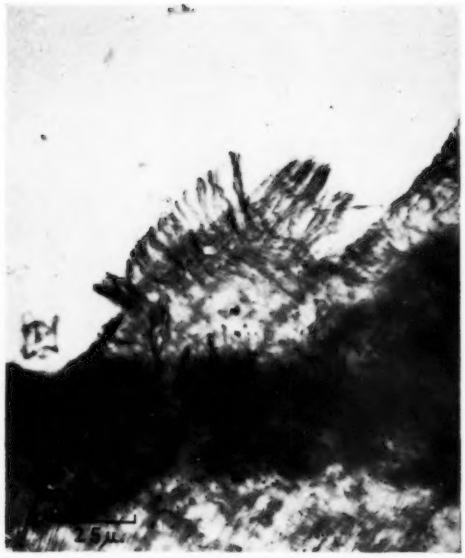

FIG. B. Cut edge of oral disk of the sea-anemone *Metridium*. Note crossed-lattice arrangement of supporting collagen fibres

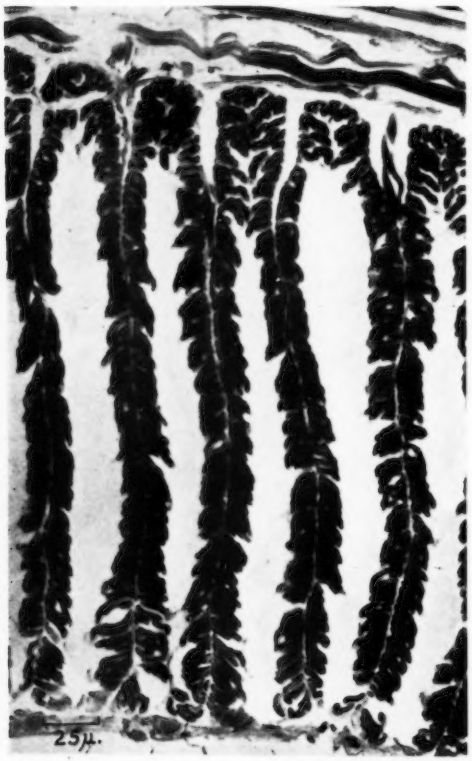

FIG. C. Transverse section of (dorsal) longitudinal muscle of earthworm *Lumbricus*, showing strap-like muscle fibres arranged on connective-tissue partitions, with free space between

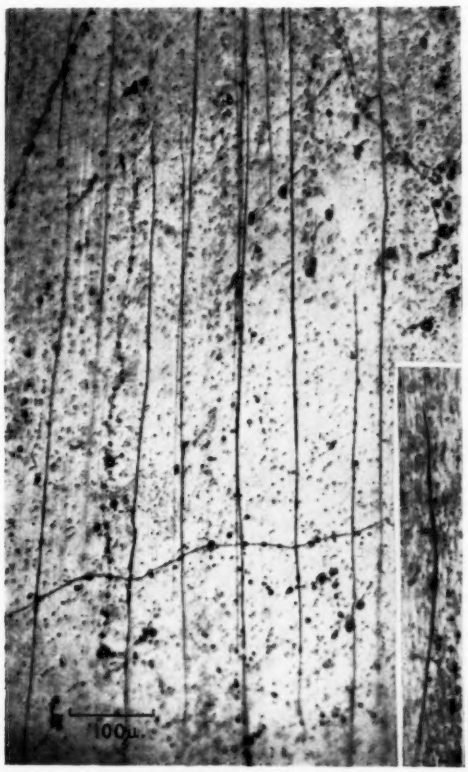

FIG. D. Retractor muscle of sea-anemone *Metridium*: a few muscle fibres stained with methylene blue. Inset: contracted muscle fibre

COMPARATIVE PHYSIOLOGY OF MUSCLE

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1 The common basis of contractility
2 The variety of muscle
3 Muscular organization
  a Functional histology
  b Functional anatomy
4 Activation of muscle
  a Tone
  b Movement
  c Relaxation oscillation
References

1. The Common Basis of Contractility

The most important conclusion that has emerged from the comparative study of muscle is that, with certain reservations, the ultimate chemical machinery underlying muscular contraction in every kind of animal is essentially the same. It has, of course, long been tacitly assumed that " muscle is muscle " wherever it is found among animals " lower in the evolutionary scale ". But even within the animal kingdom the great phyla like the arthropods and molluscs have long travelled wholly independent evolutionary paths, and are in no sense stages in the evolution of the vertebrates: and when we consider the extraordinary variety of form and function that has arisen in animals we might reasonably have expected that every class might have evolved a distinct kind of contractile machine.

All that is required for behaviour is that an organism should move; and indeed this requirement has sometimes been met in unusual ways. Some plants, like *Mimosa pudica*, move their leaves in response to stimulation by a fall in turgor. Certain beetles of the genus *Stenus* escape over the surface of water not by contractions of their limbs but by the emission from the anus of a substance which lowers the surface tension. But these are exceptions. In the overwhelming majority of cases the physico-chemical basis of movement is substantially the same (Prosser, 1950; Wiersma, 1952; Weber, 1955). In the muscles of vertebrates contraction is brought about by the agency of adenosinetriphosphate, the breakdown of which transmits energy to the contractile system. This system is not only found in muscles of very different kinds and from animals so different from the vertebrates as the insects or the molluscs, but also appears in other sorts of contractile tissue; in amoeboid cells, and in the flagella of trypanosomes. There is reason to believe that the contractile substance itself is always a complex of fibrous protein similar to the actomyosin of vertebrate muscle—even in the flagella of bacteria (Astbury, Beighton & Weibull, 1955).

This is only one example of the uniformity of the biochemical machinery of contractability. This restricted pattern of its variation is not simply of evolutionary origin, as is the pentadactyl limb of vertebrates. It depends also upon the peculiarly limited chemical materials available for living systems and the limitations set by the properties of matter, so that the same class of functional device may recur independently in unrelated organisms. These same limitations are doubtless responsible for the repeated independent evolution of similar histological structure in unrelated animals. The microscopic banded structure of striated muscle is similar not only in medusae, molluscs, insects and vertebrates, but even resembles that of the striated myofibrils of some protozoa (*Stentor*).

Structural parallelism between muscles from different animals goes hand in hand with parallelism of many mechanical properties. Hill (1926) showed long ago that, except in time scale, the development of tension in the slow unstriated longitudinal muscles of the sea cucumber, *Holothuria nigra*, resembled its development in the striated muscle of vertebrates. Muscles from very different animals share this feature (Ritchie, 1928; Abbott & Lowy, 1955).

This extensive similarity between the muscles of animals of quite distinct phyla is a fact of great importance. Were it not so, the comparative physiological study of muscle might be no more than a pleasant excursion amongst unrelated phenomena. With appropriate safeguards it justifies the invaluable assumption that experiments on the striated muscle of an insect can throw light on the nature of muscular contraction, even in man.

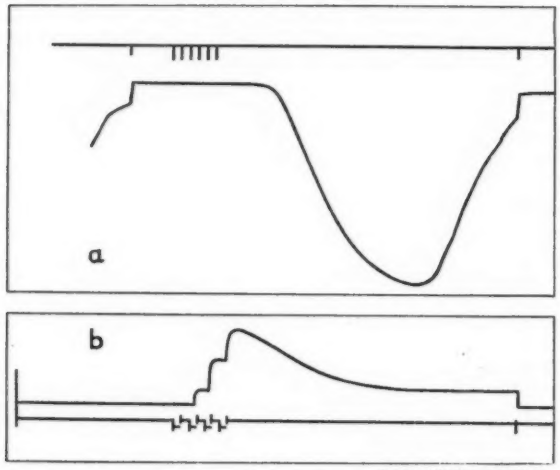
2. The Variety of Muscle

Though the ultimate machinery seems to be common to all kinds of muscle, contraction varies enormously in both speed and extent. This is true both within the Vertebrata and in other phyla. The most rapidly contracting muscles are to be found in the wing muscles of insects; less than one msec. in the fly *Forcipomyia* (Sotavalta, 1947). At the other extreme, the unstriated circular muscle of the column of the sea-anemone *Metridium senile* requires five or six minutes for a " twitch " (fig. 1a).

There is some correlation between striation and speed of contraction, though this by no means always holds. The unstriated chromatophore muscles of the squid *Loligo* execute a complete contraction and relaxation in about one-half second (Bozler, 1931). In contrast, some striated muscle contracts relatively slowly; the circular muscle of the medusa *Aurelia* takes about one second. But typically, though unstriated muscle may give a rapid contraction, relaxation is much slower, as in the marginal sphincter of the anemone *Calliactis* (fig. 1b).

The second strikingly variable property in muscle is the extent of contraction; and here there is perhaps a better correlation with histological appearance. Striated muscle is usually situated anatomically where it acts primarily by the production of tension. Plain muscle is commonly found where there is great change of length (Plate IV, fig. D). The retractor of the snail *Helix pomatia* (Bozler, 1930) can shorten about 80% of its extended length. Unlike striated muscle, which has a fairly well-defined resting length, plain muscle can be passively extended by a slight force to any resting length within this range. Such properties also occur in vertebrate plain muscle (Winton, 1926).

FIG. 1. CONTRACTION AND RELAXATION IN UNSTRIATED MUSCLE



Pantin (1950) by permission of the Company of Biologists

a: response of partly isolated strip of circular muscle of body wall of sea-anemone *Metridium* to six shocks at a frequency of one/5 sec., marked on upper line
(Note smooth contraction and very long latent period.)
b: response of marginal sphincter of sea-anemone *Calliactis* to four shocks at a frequency of one/sec., marked on lower line

3. Muscular Organization

a. Functional Histology

The operation of a muscle is governed by mechanical conditions. When a large mass of it contracts it becomes thick. The consequent deformation may be against the tension of some of the fibres, and against any viscous resistance of the tissue. This difficulty is minimized in narrow parallel-fibred muscles. In more compact muscles it may be evaded by setting the fibres obliquely on to the tendons of a moving joint, as in many arthropod and vertebrate limbs. Obliquely set fibres are able to contract without enforcing distortion of their neighbours because, though of constant volume, they can all shorten by becoming more upright, just as an oblique parallelogram can shorten when transformed into a rectangle of the same area. This principle is particularly well exemplified in the arrangement of the muscle fibres on the V-shaped myotomes of *Amphioxus* and of the vertebrates (Plate IV, fig. A).

In soft-bodied invertebrates, movement often involves great direct shortening of muscle and great consequent tissue distortion. Often the muscle fibres are extensively embedded in connective tissue (e.g. the sea cucumber, *Thyone*). Where such plain muscle fibres contract not only extensively but also quickly, the connective tissue is exceedingly loose; as in the well-developed longitudinal muscle system of the earthworm *Lumbricus terrestris* (Plate IV, fig. C). The rows of strap-like muscle fibres lie in fluid spaces within sparse connective tissue.

The connective tissue about plain muscle is of considerable mechanical importance. The muscle sheets of the body wall of sea-anemones are attached to a layer of connective tissue, the mesogloea. This tissue is able to follow the astonishing changes of shape which these animals undergo, because it consists of fibres arranged at 45° in a well-defined system of crossed lattices (Plate IV, fig. B) (Chapman, 1953a; Pantin, 1952). As in the case of " lazy tongs ", such lattices have great extensibility. Similar lattices occur in the connective tissue of the body wall of many worms, such as the highly contractile Nemertinea (Cowey, 1952), and possibly in the viscera of vertebrates.

b. Functional Anatomy

Most animals move by differentiated muscles. This is clearest in the extensors and flexors of jointed limbs. It is also true of many soft-bodied animals, though here the operation of the muscle is more direct. The mussel *Mytilus* is directly pulled towards its attachment to the rocks by its long " byssus retractor ".

But in soft-bodied animals, including most of the simplest and most " primitive ", the shape of the body and its movements depend on the contraction of a muscular body wall acting against the volume of fluid or soft tissue within. Here, the relation of contraction to movement is complicated. It is partly direct, as in the retraction of a tentacle in hydra; but it is equally enforced indirectly through internal pressure, as in the elongation of a sea-anemone through the contraction of the circular muscle layer of its body wall. The mechanics of these soft-bodied animals are of general interest; even in vertebrates the movement of viscera like the gut or the uterus involves the same principles. Batham & Pantin (1950) analysed the mechanics of movement in the sea-anemone *Metridium*. These animals consist of a muscular body wall surrounding a stomach full of sea-water. The shape is changed by squeezing the sea-water. The system works at a very low pressure. Pressures of 2–7 mm. of water accompany the normal changes of shape. The maximum pressure that can be set up by the body-wall muscles is about 12 mm. of water. At this pressure the muscles are acting isometrically and, being unable to shorten, they cannot change the shape. Almost all the natural change of shape is executed against pressures between 10% and 50% of the isometric. Mechanically this is the most efficient range.

Clearly, the " isometric ceiling " depends upon the work the animal has to do in its natural habitat (see Table I). It is much greater for burrowing worms than for the sea-anemones in open sea-water. Animals that burrow in earth and sand must work at a much higher pressure and have a much higher isometric ceiling. Animals that live in crevices in rocks have an intermediate pressure range.

TABLE I. RELATION OF INTERNAL PRESSURE DEVELOPED, IN SOFT-BODIED ANIMALS, TO THEIR NATURAL HABITAT

Animal	Maximal internal pressure (mm. H_2O)	Average normal pressure (mm. H_2O)	Habitat	Reference
Metridium senile	12	2–7	Open sea	Batham & Pantin, 1950
Holothuria grisea	200	7–21	Crevices	Pantin & Sawaya, 1953
Caudina chilensis	450–500	*ca* 150	Sand	Yazaki, 1930
Sipunculus nudus	960	1.5–70	Sand	Zuckerkandl, 1950
Arenicola	800–900	*ca* 130	Sand	Chapman & Newell, 1947

4. Activation of Muscle

a. Tone

The large and prolonged tensions set up in the muscles of many invertebrates, particularly Mollusca, led to the suggestion that there might be two distinct mechanisms present: that of a clonic twitch, and that in which the muscle assumes a passive state of rigidity in which it can sustain great weights—a state which is initiated and terminated, but not maintained, by nervous action. Bayliss, Boyland & Ritchie (1930) pointed out that most, perhaps all, of these phenomena could be explained by the very slow and " viscous " action of the twitch in such muscles, whereby a tetanus can be economically maintained with very little nervous and metabolic action. Bozler (1948) has come to a similar conclusion, and recently Lowy (1953, 1954), by the direct recording of action potentials of molluscan muscles (*Mytilus*, *Pecten*) has shown with great clarity that much of the tonic behaviour of these muscles is in fact of this nature.

Particularly in this sort of muscle, there is another factor of importance in tone. The extensibility of muscle depends not only on the contractile fibres, but also on sarcolemma and the surrounding connective tissue to which these are attached. Indeed many of the " plastic extensibility " phenomena that have been recorded in invertebrate plain muscle are really properties of the connective tissue and occur even if the muscle fibres themselves are out of action (Chapman, 1953b; Batham & Pantin, 1951). A twitch may be rapid because neither contraction nor relaxation has time to deform the underlying connective tissue; whilst a prolonged contraction may result in a slow deformation which prevents rapid relaxation.

Even so, although the original " rigidity " hypothesis cannot be sustained, " viscous " tetani and deformation of connective tissue will not account for all the phenomena. Twarog (1954) showed that tone can be induced in *Mytilus* byssus retractor by acetylcholine. This is not a tetanus, though Abbott & Lowy (1955) have shown that it is accompanied by heat production.

Sometimes the rate of relaxation can be accelerated by nervous action. There is active nervous inhibition of the contractile state in the closing muscles of several bivalve molluscs (Benson, Hays & Lewis, 1942; Lowy, 1954). Some chemical substances have striking effects. Winton (1926) found adrenaline greatly increased the rate of relaxation of the retractor penis of the dog, whilst Twarog (1954) has shown that, in the byssus retractor of *Mytilus*, 5-hydroxytryptamine causes rapid relaxation of tone. Recent work on inanimate fibrous systems has shown how greatly rate of extension can be affected by plasticizing substances. Such might affect the rate of relaxation of muscles: and the studies we have just discussed raise the interesting possibility that such action might conceivably be on the connective tissue as well as on the contractile fibres.

b. Movement

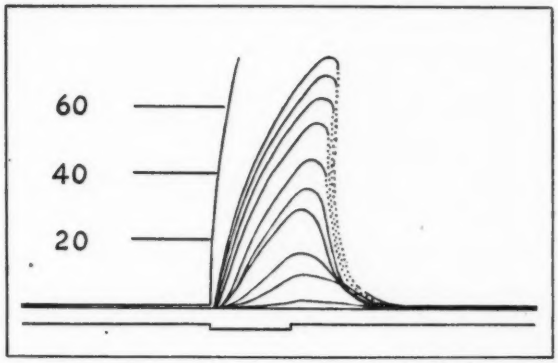
In the mammals, graded contraction of voluntary muscle takes place through control of the number of active motor units. Each unit is served by one motor nerve cell, and under normal conditions each impulse in the nerve cell directly excites all, or nearly all, the muscle fibres of the unit and does so in an all-or-nothing manner. Gradation of control is thus central. In contrast, nervous control of visceral muscle is complex, often involving autonomous muscular activity and controlled by both excitor and inhibitor nerves.

The mammalian motor unit is not a primitive unit of animal neuromuscular action. It is a good example of the secondary evolution of an efficient simplicity (Pantin, 1950, 1951). In some vertebrates we find in fact a more complex system; particularly in the frog, alongside the usual motor units, there is a separate system of slower muscle fibres innervated by axons of small diameter. They do not excite all-or-nothing responses in the muscle fibre, but localized contractions (Kuffler, 1953). This system shows some parallel to that found in many invertebrate animals (Wiersma, 1952).

Control of contraction by gradation of motor units seems to be peculiar to the vertebrates. Many animals have evolved a system in which an axon excites a muscle fibre to all-or-nothing response, but outside the vertebrates this property is restricted to the total activation of an entire muscle. It is particularly evident in muscles concerned with escape, as in the tubicolous worms (e.g. *Branchiomma* (Nicol, 1951)) in which the longitudinal muscle suddenly retracts the animal into its tube. There is no gradation in such responses.

In most invertebrates, graded response is effected in a different way. The sphincter which closes and protects the mouth and disk of the sea-anemone *Calliactis parasitica* is innervated by a specialized nerve net, the whole of which acts as a single unit conducting a nervous impulse throughout its extent. A single nervous impulse causes no contraction, but it leaves a facilitating effect so that a subsequent impulse following within one or two seconds causes the muscle to contract (fig. 1b). The facilitating effect decays, so that the size of the contraction depends on the frequency of the impulses; impulses at short intervals cause a big response, while those at longer intervals produce a small one, and below a certain frequency none at all (Pantin, 1952). In most invertebrates (e.g. crustaceans, insects, molluscs and annelids) the muscles are served by very few motor axons, and the response is graded according to the frequency of impulses conducted to the muscle (fig. 2). The range of frequency varies greatly. In contrast with the low frequency of facilitation in sea-anemones, the frequency of impulses reaching

FIG. 2. SUPERIMPOSED CONTRACTIONS OF FLEXOR OF DACTYLOPODITE OF LEG OF CRAB *CARCINUS*



Pantin (1936) by permission of the Company of Biologists

Duration of stimulus: 0.45 sec.

Frequencies of stimulation/sec.: 250, 185, 137, 103, 94, 79, 67, 51, 44, 33

Vertical scale: isometric tension developed (g.)

crustacean limbs reaches hundreds per second. It must not be assumed that all facilitation mechanisms are the same.

Facilitating systems have been very thoroughly investigated in Crustacea (Katz, 1949; Wiersma, 1952). Despite the few axons serving the muscle, the system here is highly complex. Commonly the muscle is innervated by two distinct kinds of motor fibre and by an inhibitor as well. The smaller motor nerve fibres produce small local potentials on the muscle fibres, similar to the end-plate potentials (e.p.p.) in vertebrate muscle. These are accompanied by localized contraction of the muscle fibre, but no all-or-nothing contractions. Repetitive stimulation causes increase by facilitation of both the size of the e.p.p. and the size of the local contraction. Appropriate stimulation of the inhibitor nerve can inhibit by reducing the e.p.p. and by increasing its rate of decay.

The action of the larger motor fibres differs with the species of crustacean. In some slowly moving ones, their excitation causes facilitated contractions differing from that in the small motor fibre system only in their greater speed of development. But in the claws of *Astacus*, stimulating the large motor fibres of the " quick " system produces an all-or-nothing twitch of the whole muscle without facilitation. Katz & Kuffler (1946) have produced evidence that here these large e.p.p. initiate an action current propagated along the muscle fibres.

Insects, molluscs, annelid worms and echinoderms show some evidence of muscular control by facilitation, though their mechanism has not yet been worked out in as much detail as it has for the crustaceans. Control of speed and extent of contraction of limb muscles in such animals is as efficient as it is in vertebrates. The important difference is that the motor unit system of vertebrates allows control to be central, whereas in other animals it is peripheral.

This peripheral excitor and inhibitor control shows some resemblance to that of the vertebrate autonomic system. Indeed in worms and sea-anemones, autonomic activity itself is an important element in active behaviour (Wells, 1955).

c. Relaxation Oscillation

Pringle (1949, 1954) has recently shown that the rapid oscillations of the wings of the blowfly *Calliphora* are produced on a wholly novel principle. The contractions of the muscles take place at a high frequency of several hundred per second. This is not due to separate contractions following the arrival of each impulse. Motor nerve impulses of only moderate frequency excite the muscle system in such a way that the whole structure is set into high-frequency oscillation. This takes place because the box-like thorax to which the muscles are attached is so constructed that it can, in effect, " click " in and out rather after the fashion of a dented tin; it moves the wings in so doing. The muscle is in fact in a state of continuous excitation, but the sudden release of tension during each sudden " click " deactivates the myofibrils so that the tension abruptly falls. The deformation of the thorax is now restored by contraction of the antagonistic flight muscles. These in their turn are thereby inactivated; and by now the first set of myofibrils have redeveloped their active state, and the cycle proceeds. This remarkable device has apparently arisen independently in more than one group of insects, and in a simpler form in the " singing " mechanism of the cicadas. In principle, the same trick by which a wrestler takes advantage of his opponent by suddenly releasing the tension of his muscles is here used for an ingenious and very different end.

* * *

A survey of muscular action in the animal kingdom shows how limited are the materials and the classes of machine available for the production of movement. Repeatedly, similar physiological solutions are evolved to meet the functional problems which beset the animal. But no particular solution is necessarily barred. Occasionally some special chemical or mechanical property is exploited to give an unusual and seemingly highly ingenious solution of a functional problem.

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MUSCULAR ACTIVITY DURING LOCOMOTION

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1 The movement of fish
2 The mechanics of standing in four-footed animals
3 Four-footed movement
4 The starting and checking of movements
5 The physiological integration of movement
References

A complete picture of the muscular activity of a moving animal would involve a detailed knowledge of the changes in tension and length of a very large number of muscles and a very precise knowledge of their anatomical arrangement. In practice it is exceedingly difficult to obtain this information concerning even one muscle in situ without interfering with the animal's freedom of movement; to extend such observations to a large number of muscles is quite impracticable. A more useful—though less complete—picture emerges if the entire animal is regarded as a single unit whose over-all mechanical effort is expressed in terms of the forces which it exerts against the outside world. These forces can be measured with considerable accuracy and from them it is possible to assess the muscular effort being applied about each of the constituent joints of the body.

The ability of a vertebrate animal to exert a thrust against its external environment depends on its power to operate its bones as levers, but before applying this very familiar concept to the problems presented by a moving animal it is useful to bear in mind two rather obvious, but important, facts. First, a muscle cannot exert tension against a bone without at the same time exerting an equal but opposite force against the bone to which the other end of the muscle is attached; second, a muscle cannot exert tension against either of these bones without inducing a corresponding degree of compression between the two surfaces of the joint across which it acts. In other words, the mechanical effect of an active muscle is the application of equal but opposite turning couples to the two bones, the moment of the couples depending on the tension of the muscle and its distance from the centre of rotation of the joint.

1. The Movement of Fish

The chain of levers which constitute the working parts of a vertebrate's propulsive machine is seen in its simplest form in fishes; in these animals the vertebrae are so arranged that the axis of each can be deflected laterally with respect to those of its neighbours, and all these movements are effectively restricted to a single lateral plane. The mechanical principle of such a system is shown in fig. 1, in which the tail of the fish is shown sweeping from left to right under the force exerted by the contraction of the muscles of the right side of the body; the tail and caudal fin are bending under the pressure exerted by the surrounding water. The force (R_T) exerted against the tail can be measured with reasonable accuracy. In order that the caudal fin should elicit this reaction from the water the muscles (m) operating about any particular vertebral joint (h) must exert an anti-clockwise turning couple (C_1, T_1) on the region of the body posterior to h, the moment ($R_T x$) of this couple being equal to that of the force R_T about the same hinge. At the same time, the muscles exert an equivalent clockwise turning couple (C_2, T_2) on the region of the body anterior to the hinge. The external forces acting on the hind end of the body are the anti-clockwise muscular couple and the reaction (R_T) of the water against the caudal fin; the resultant of these is a force (F) equal and parallel to R_T but operating against the hinge. The force F has two components: one of these (P) propels the body forwards, the other (L) tends to rotate the front end of the body about its centre of inertia and to displace this part of the body towards the left. If the head of the fish is to move steadily forward without oscillating from side to side, the front end of the body must be restrained from the turning movements which would be induced by the force L and by the muscular couple; in nature, this restraint is provided partly by the high inertia of the front end of the body and partly by the lateral pressures exerted by the water; these two factors reduce lateral oscillations of the head to relatively small amplitudes. A model working on these principles is shown in fig. 1e and fig. 2.

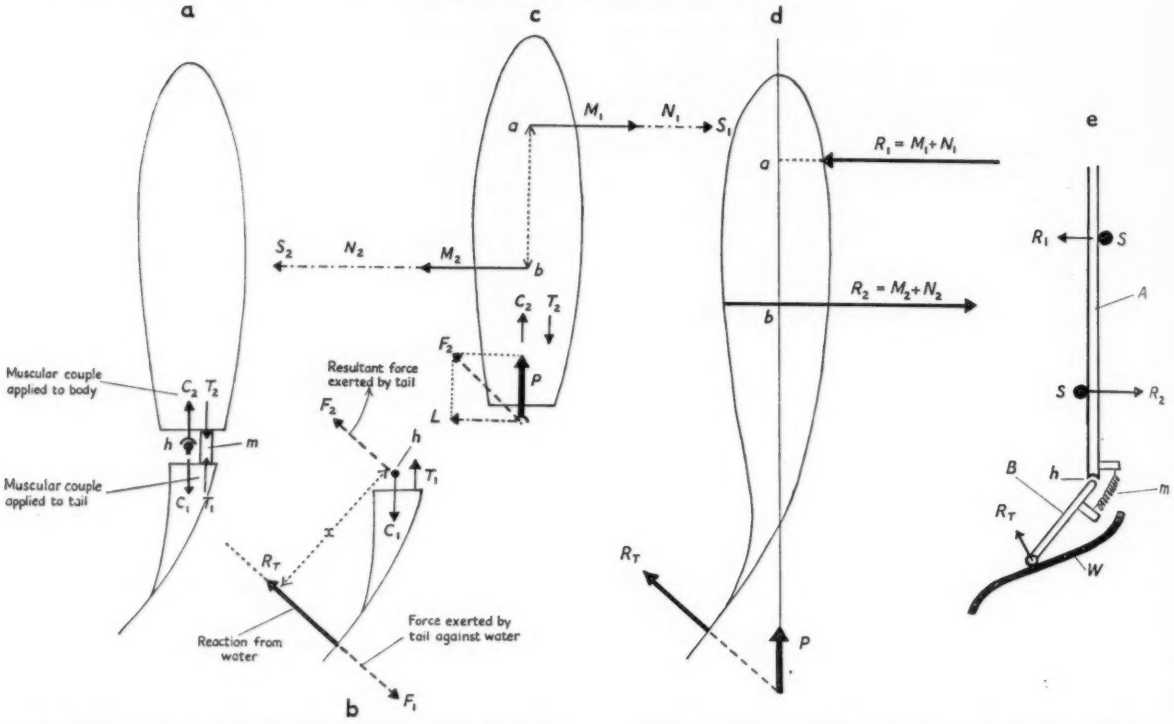
Before applying a similar type of analysis to the locomotion of other types of vertebrates, it is perhaps worth noting the general level of muscular activity that can be reached by a moving fish as expressed in terms of external effort. When swimming steadily very few, if any, fish can maintain by muscular effort a propulsive thrust of more than a small fraction of their total weight; they can, on the other hand, exert several times their own weight for very brief periods. The muscular equipment of a typical fish is designed for rapid acceleration; once the animal is under way, a relatively small effort is required to maintain movement. At first sight it seems difficult to accept the fact that the powerful effort which has to be exerted against a rod by an angler playing a large, fast, running, fresh salmon exerts a tensional strain against the fish of not more than about one pound[1] and often less. Even a powerful fish tires under this slight restraint relatively quickly. When a salmon breaks a cast whose limiting strain is many times the fish's own weight, the fracture is due to the inertia of the fish's body and not to forces exerted by its muscles; to bring a 20-pound salmon travelling at about 5 miles[2] per hour to rest within 3 feet[3] would involve an average tensional strain of about 160 pounds.

2. The Mechanics of Standing in Four-Footed Animals

As contrasted with those of a fish, the range of posture and movement available to a terrestrial tetrapod are largely determined by the overriding effect of the animal's own weight (Gray, 1944, 1953). The body of a dog or horse can be regarded as a flexible beam supported and propelled by the four elastic legs. To sustain the weight of the body, a limb must exert a vertical thrust, and to drive the body forward it

[1] 1 pound = 453.59 g.—Ed.
[2] 1 mile = 1.6 km.—Ed.
[3] 1 foot = 30.48 cm.—Ed.

FIG. 1. RELATIONSHIP BETWEEN THE FORCE PROPELLING A FISH AND THE FORCES EXERTED BY THE LATERAL MUSCULATURE



a: the muscles (m) on the right side of a vertebral hinge (h) are under tension and are applying a clockwise turning couple (C_2, T_2) to the front end of the body and an equal but opposite couple (C_1, T_1) to the tail and caudal fin. The tail of the fish is moving towards the right side of the body.

b: the pressure (F_1) exerted by the tail against the water elicits the reaction (R_T) acting normally to the surface of the fin, and the moment ($R_T x$) of this force about the hinge is equal to that of the muscular couple (C_1, T_1). The resultant of the couple and the force R_T is F_2 equal and parallel to R_T acting at the surface of the hinge against the front end of the body.

c: the force F_2 has two components: one (P) drives the fish forwards, the other (L) tends to rotate the front end of the body and to displace it to the left. The same mechanical effect as L would result from two lateral forces: N_1 acting at a and N_2 acting at b. Similarly, the effect of the muscular couple (C_2, T_2) is equivalent to lateral forces (M_1 and M_2) also acting at a and b. The resultant forces (S_1 and S_2) tend to produce turning and lateral displacements.

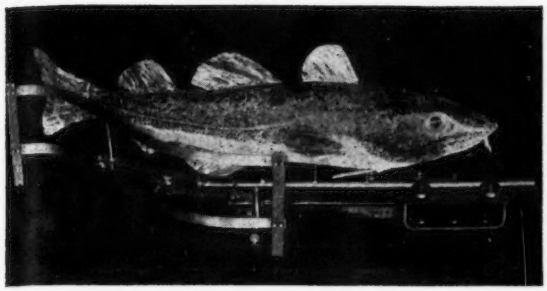
d: if the head of the fish is to move forwards without oscillating from side to side, the front end of the body must be subjected to external lateral forces (R_1 and R_2) equal but opposite to S_1 and S_2, the resultant of R_1 and R_2 being equal but opposite to the lateral component (L) of the force (R_T) exerted by the water against the tail.

e: principle of the working model shown in fig. 2. The front end of the body is represented by the rod A, the tail and caudal fin by rod B. The muscles are represented by a spring, and the water, pressing against the tail, by a rigid slide (W). The rod A is prevented from lateral displacement by the stops (S). If the spring develops tension the model slides forward.

must exert a suitably directed thrust along the vertebral column. The mechanical properties of a limb are illustrated by fig. 3. In fig. 3a both fore- and hind-limbs are acting as extensible struts in the sense that the force exerted by the foot against the ground passes through the centre of rotation of the joint which unites the limb to the body. As drawn in the figure the mechanical axis of the fore-limb is vertical, and only a vertical upward thrust is being applied to the body; the axis of the hind-limb is, however, inclined to the vertical, and consequently the axial thrust applied to the body has a horizontal propulsive component. In neither limb as shown in fig. 3a does any differential strain fall on the muscles operating about the hip or shoulder joints. In both cases the limb is acting as a strut, and its ability to function as such is determined by the muscles operating about the internal joints of the limb; as a strut the limb's main function is to support the body against gravity, but it may—with a suitably inclined posture—exert a horizontal propulsive thrust or act as a brake. In all cases the muscular couples operating about a particular joint can be derived from a knowledge of the force which the limb exerts against the ground and of the position of the joint relative to the line of action of this force.

In addition to acting as a strut, the limb can function as a lever by utilizing the muscles operating between the limb and the body. This principle is illustrated in fig. 3b. The moment of the muscular couple exerted by the hip muscles or shoulder muscles is again determined by the magnitude of the force exerted by the foot and the distance of the relevant joint from the line of action of this force. It is useful to notice that the force exerted by a limb as a lever is almost completely restricted

FIG. 2. WORKING MODEL OF PROPULSIVE MECHANISM



to a horizontal plane. In other words, the function of support is mainly performed by the intrinsic muscles of the limb, whereas the function of propulsion is performed by the extrinsic muscles aided by the intrinsic muscles if the axis of the limb is suitably inclined to the vertical.

During normal locomotion the support and propulsion of the body is performed by four limbs, and during each cycle of limb movements the lengths and tension of all the muscles are changing. Before considering a system of such very great complexity, it is convenient to consider the general principle of co-ordinated limb effort which emerges from a study of the forces which operate when the animal is at rest. In fig. 4 the system is reduced to its simplest form; all four limbs are functioning as vertical struts. The mechanical stability of the body depends on the fact that the resultant of the forces exerted by the limbs against the shoulders and hips is exactly equal but opposite to the weight of the body acting through its centre of gravity; in fig. 4a the centre of gravity is assumed to be equidistant from the front and hind-feet. So long as the whole of the animal is at rest the vertical thrust from the limbs must satisfy three equations:

$$T_{LF} + T_{RF} + T_{LH} + T_{RH} = W \quad . \quad . \quad \text{(i)}$$

$$\frac{l}{2}(T_{LF} + T_{RF}) = \frac{l}{2}(T_{LH} + T_{RH}) \quad . \quad . \quad \text{(ii)}$$

$$\frac{t}{2}(T_{LF} + T_{LH}) = \frac{t}{2}(T_{RF} + T_{RH}) \quad . \quad . \quad \text{(iii)}$$

when T_{LF}, T_{RF}, T_{LH}, T_{RH} are the vertical thrusts exerted by the left fore-, right fore-, left hind-, and right hind-limb respectively; W is the total weight of the body; l is the distance between the front and hind-feet and t is the distance between the right and left feet. From these three equations it can be seen that half the total weight of the body must be carried by the two front feet and half by the two hind-feet. Similarly, half the weight is carried by the two left feet and half by the two right feet.

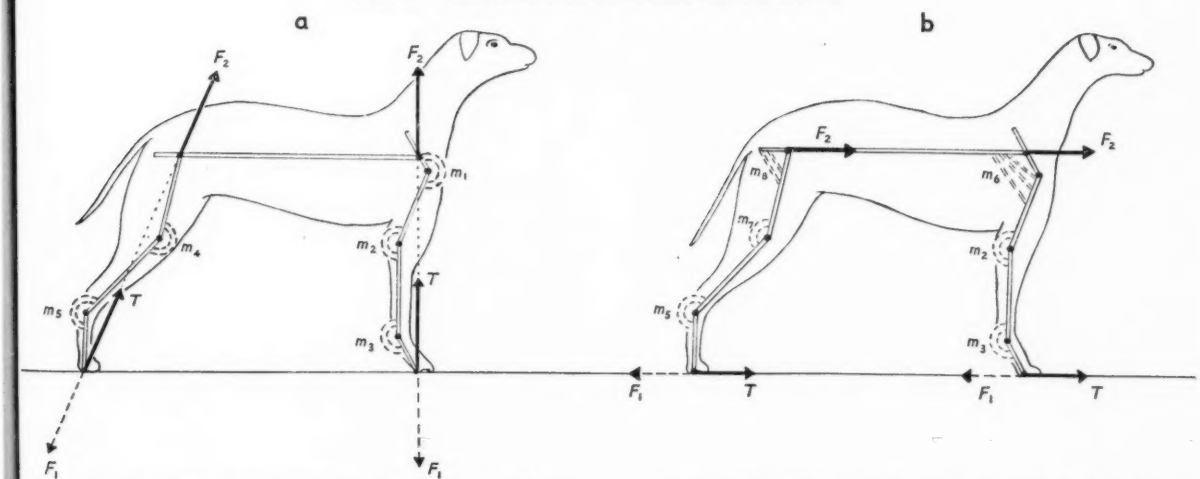
A more significant deduction can be drawn by ascribing a particular value for the thrust of a specific limb, thus enabling the thrusts of the other three limbs to be calculated. For example, if the thrust of the left fore-limb be x, then the thrusts of each of the other three must be as follows:

$$\text{Right fore-limb } (T_{RF}) = \frac{W}{2} - x \quad . \quad . \quad \text{(iv)}$$

$$\text{Left hind-limb } (T_{LH}) = \frac{W}{2} - x \quad . \quad . \quad \text{(v)}$$

$$\text{Right hind-limb } (T_{RH}) = x \quad . \quad . \quad . \quad \text{(vi)}$$

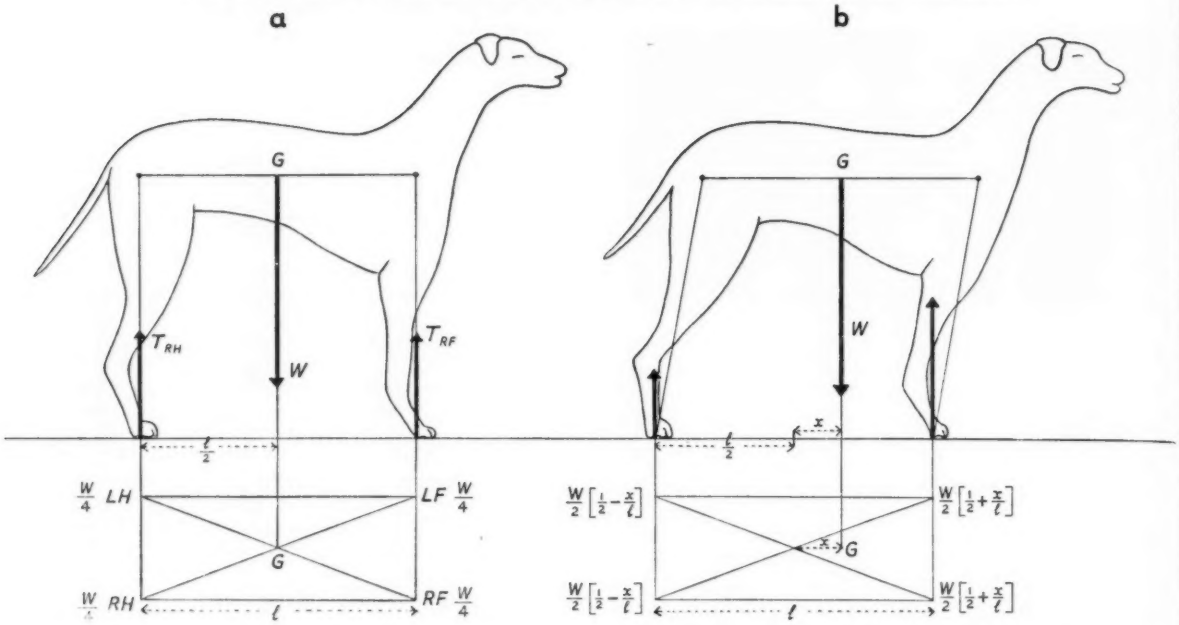
FIG. 3. MECHANICAL FUNCTIONS OF LIMBS



a: both limbs functioning as struts. The equal but opposite forces (F_1, F_2) which the limb exerts against the ground and against the body act along the limb's mechanical axis, i.e., along the line joining the centre of pressure of the foot to the centre of rotation of the joint by which the limb is attached to the body. In the figure the axis of the fore-limb is vertical and only a vertical force is applied to the body; the hind-limb is inclined and the body receives a forward propulsive thrust as well as a vertical supporting force. The active muscles (m_1–m_5) are shown by dotted lines; no differential strain falls on the retractor or protractor muscles of the shoulder or hip.

b: both limbs operating as levers. Couples are developed by the retractor muscles of the shoulder and hip (m_6, m_8). The forward thrust applied to the body is equal to the friction (T) acting against the feet. Note that the activity of the shoulder and hip muscles necessitates appropriate changes in the activity of the intrinsic muscles of the limbs (m_2, m_3, m_5, m_7).

FIG. 4. GENERAL PRINCIPLES OF SUPPORT AND PROPULSION OF THE BODY



a: the centre of gravity (*G*) of the animal shown lies midway between the shoulders and hips; the limbs are vertical. If the vertebral column is to be free of all torsional strain, the vertical forces exerted by each limb in its function as a strut must be the same for all four limbs (*LH, RH, LF, RF*)—each carrying one-quarter of the total weight of the body (*W*), as shown in the lower part of the diagram. The centre of gravity of the body is vertically above the intersection of the lines joining diagonal limbs. T_{RH} and T_{RF} are vertical thrusts exerted by the right hind- and right fore-limb respectively, and *l* is the distance between the front and hind-feet.

b: the animal has moved its shoulders and hips forward through a distance *x* relative to its feet. To enable the animal to stand in this position, with its back still free from torsion, the limbs must be operated as levers and their vertical thrusts adjusted to the values shown in the figure. The animal can lift either of its hind-feet provided (i) the muscles of the back develop adequate torsional couples; (ii) the thrust of the diagonal fore-foot is reduced by an amount previously exerted by the lifted hind-foot; (iii) the thrusts of both limbs of the other diagonal limbs are increased by a corresponding amount. If the left hind-foot is lifted, the thrust of the right fore-limb must become $\frac{W}{2}\left[\frac{2x}{l}\right]$, that of the left fore-limb $\frac{W}{2}$, and that of the right hind-limb $\frac{W}{2}\left[1-\frac{2x}{l}\right]$.

Two interesting facts emerge: (i) the thrust of any one limb (for an animal in the posture shown in the fig. 4a) is always equal to that of the diagonal limb on the other side of the body; (ii) any decrease in the thrust exerted by one limb must be accompanied by a similar decrease in that of its diagonal partner and by a corresponding increase in that of each of the limbs on the other diagonal. In other words the well-known "crossed extensor" reflex receives a functional meaning in terms of purely mechanical factors. Equations iv–vi also illustrate another fact of some physiological interest, for they show that the thrust exerted by any one of the four limbs can vary from $\frac{W}{2}$ to zero, without involving instability of the body except at the extremes of this range. On the other hand, unless the weight of the body is equally distributed between each of the four limbs, the vertebral column must actively resist torsion: the greater the difference in the distribution of weight between two front or two hind-feet, the greater is this torsional strain. Putting matters in a different way, an animal can adjust the distribution of weight between its limbs by controlling the activity of the torsional muscles of its vertebral column. Alternatively, if the animal shown in the fig. 4a wishes to avoid exposing its back to torsional strain, it must adjust the activity of its intrinsic limb muscles in such a way as to ensure that the total weight of the body is equally distributed between each of its four limbs. For present purposes, however, it is sufficient to note that even when an animal is standing quietly at rest, any voluntary change in the tensile activity of one muscle involves a change in the activity of nearly every other somatic muscle in the body. The response to a "flexor" stimulus applied to one limb must spread far beyond the extensor muscles of the contralateral limb; the whole of the somatic musculature must be regarded as a single functional unit.

3. Four-Footed Movement

Bearing in mind the mechanical conditions necessary to maintain the stability of an animal whose body is at rest, it is possible to consider the nature of the muscular effort required to propel the body forward in an orderly way. Again, it is convenient to start with an animal standing with the mechani-

cal axis of all its limbs vertical. In such a posture, the animal can develop a forward thrust against its body only by exercising one or more limbs as a lever operated by the retractor muscles which unite the limb or limbs to the body. If this effort is restricted to one limb (e.g. right hind), the resultant horizontal thrust will not pass through the centre of gravity of the body and consequently the body will tend to swing or yaw towards its left side; this disturbance will be compensated if the retractor muscles of the diagonal limb (e.g. left fore) also develop an adequate propulsive force against the other side of the body. In other words, smooth regular translatory movements can be performed by the body only if there is a degree and type of co-ordination between the muscles of diagonal limbs of the same general type as that necessary to maintain the axis of the body in a fixed horizontal plane.

As soon as the body of an animal (similar to that shown in the fig. 4a) begins to move forward relative to the feet, an important change occurs in the position of the centre of gravity relative to the fore- and hind-feet (fig. 4b); instead of being equidistant to these two pairs, it now lies nearer to the front feet and within a triangle demarcated by the two front feet and one of the hind-feet. In other words, the animal can now lift one hind-foot off the ground altogether without falling over: it cannot lift a front foot, although it can use both of them and the remaining hind-foot for supporting and propelling the body. For stable progression, the centre of gravity of the body must lie within the triangle of support of the three feet on the ground during the whole of the period during which the fourth foot is being swung forward ready for its next step.

If three feet are always to be on the ground, it follows that during each complete cycle of its movements each limb must be off the ground for a quarter of the duration of the cycle and on the ground for three-quarters. As, at the same time, the centre of gravity of the body must always lie within the triangle of the three supporting feet it follows—from purely geometrical principles—that the feet must be lifted from the ground in a particular order, viz. left fore—right hind—right fore—left hind. This order is usually referred to as the "diagonal sequence", and is highly characteristic of all quadruped animals when walking slowly but steadily; it is, in fact, the only pattern of movement in which the body is statically stable at all phases of the walk.

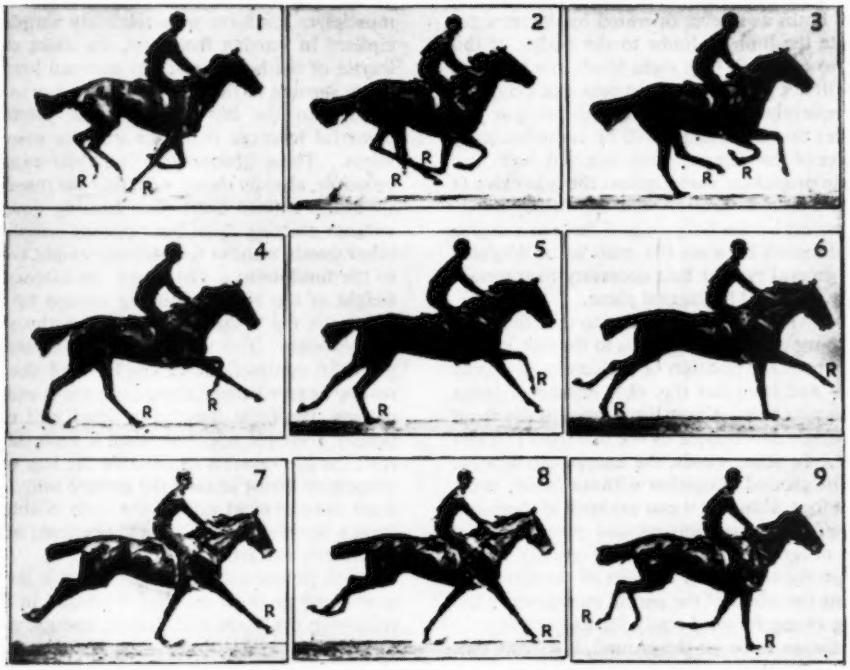
As the pace of an animal quickens, each foot is lifted slightly before the one next ahead of it in the sequence has reached the ground, and there are phases of movement in which only two limbs are on the ground instead of three. With still greater increase of speed, the periods during which three limbs are on the ground becomes shorter and shorter until eventually only two limbs support and propel the body simultaneously; usually diagonal limbs work together, as in a trot; but sometimes, as in a rack, the two ipsilateral limbs collaborate. In the fastest gait—or gallop—the two fore- and two hind-limbs function more or less simultaneously. In all cases, however, the centre of gravity of the body falls during certain phases of progression and has to be raised again when the appropriate foot or feet can again exert an active thrust against the ground.

4. The Starting and Checking of Movements

Within the limits of this article, it is impossible to consider more than a few general characteristics of the muscular effort involved by the locomotion of an animal such as a horse or of a man, but a few easily observed phenomena illustrate the dominating extent to which the organism adjusts the use of its muscles to conform with relatively simple mechanical principles. In starting from rest, the main effort is against the inertia of the body or of any external load. For example, a horse starting to move a heavy van moves its feet backwards relative to the hip and shoulder joints before applying powerful leverage from the muscles operating about these joints. These phenomena find their explanation from the principle, already mentioned, that the muscles of the hips and shoulders cannot exert their turning couples on the limbs without exerting equal but opposite couples on the body—in other words without transferring weight from the front limbs to the hind-limbs. The larger the extent to which the total weight of the body is initially carried by the front feet, the greater is the horizontal propulsive thrust which the limbs can develop. The upward tilt of the body in response to powerful contractions of the hip and shoulder muscles can readily be seen when a standing horse is suddenly spurred into motion, the front legs being lifted well off the ground. A similar principle operates when a man starts to sprint from rest; the hip muscles can enable the legs to exert a powerful propulsive thrust against the ground only if the couple which these muscles exert against the body is able to raise the body from a horizontal to a vertical position; hence, the sprinter's characteristic starting posture.

When an animal starts from rest, a large fraction of the tensile energy of its muscles is utilized in the useful work of endowing the body with kinetic energy, and so long as the translatory speed is rising some useful work is done. Eventually a maximum speed is reached and at this point all the energy of the muscles is being dissipated against internal resistances, except the very small fraction required to overcome the resistance of the surrounding air. To some small extent the increase in internal resistance associated with high translatory speeds may be due to increased frictional forces within the muscles and the joints, but to a very much greater extent it is due to the inertia of the limbs themselves which must be kept moving relative to the body. For example, when a horse is running steadily at 30 miles per hour, the horizontal forces exerted by the feet against the ground are negligibly small compared with those operating when the speed is rising relatively rapidly. In a steadily moving animal the main function of the limbs is to prevent the body falling under gravity; in fulfilling this role they do very little active work but they consume a very considerable amount of energy. The movement of the limbs of a running horse is illustrated by fig. 5. This figure shows that, although a limb is fully protracted at the end of its forward swing, it does not establish contact with the ground until the hoof is nearly vertically below the hip or shoulder; at this stage the hoof is travelling backwards relative to the hip or shoulder at the same speed as the body is travelling forwards relative to the ground; in other words, at the moment of contact the hoof is stationary relative to the ground and can exert a vertical thrust without eliciting any frictional resistance from it. In order to establish these conditions, the force applied to the fully extended limb must be such as will give the limb sufficient acceleration to raise the speed of the hoof to 30 miles per hour within a distance equal to about one-half of the limb's natural swing. A rough estimate of the acceleration required is about six times that of gravity. During the whole period of contact the limb must continue to swing backwards relative to the body

FIG. 5. SUCCESSIVE PICTURES OF A GALLOPING HORSE



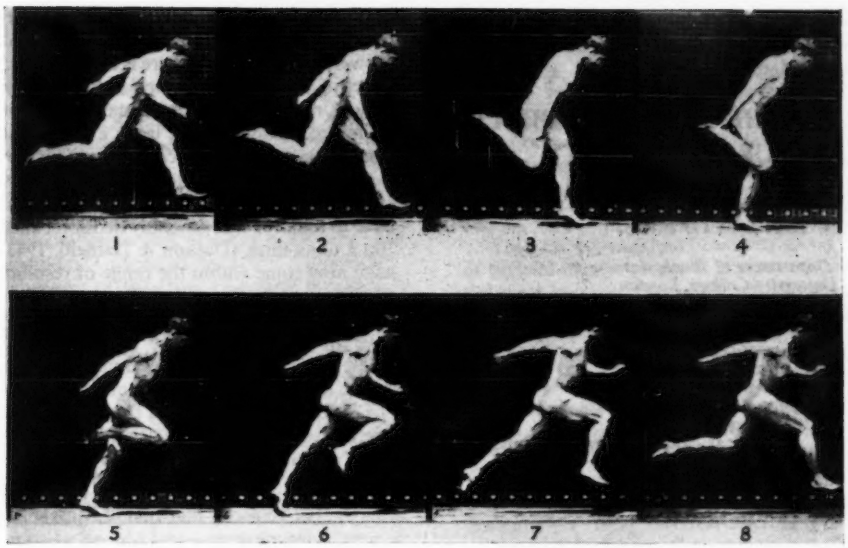
Muybridge (1899) by permission of Chapman & Hall

with unreduced speed; fig. 5 (7) shows that the backward swing continues after the hoof leaves the ground. To check the backward swing of a limb before the hoof has left the ground would operate the limb as a brake; to check it immediately after it leaves the ground would involve an intense effort by the retractor muscles just before they are being called upon to swing the limb forward. In practice the horse allows its limb to complete its backward swing after the foot has left the ground on the same principle as the club and shoulders of a golfer's " follow through " after the ball has been struck. In both cases the danger of checking the speed of the limb or club too soon is avoided. As soon as the limb has come to rest in the fully retracted position it has to be swung forward again ready for the next step. The speed at which the protractor muscles can accomplish this depends on the moment of inertia of the limb. Figure 5 (3) shows how the work of the protractor muscles is minimized by sharp flexure of the limb and consequential reduction of its moment of inertia. Precisely similar phenomena are noticeable in human running (fig. 6). It would be interesting to know how far the difference between an expert and an indifferent athlete depends on the possession of more powerful muscles and how far on the conscious or unconscious ability to use his muscular energy to its best physical advantage.

5. The Physiological Integration of Movement

The study of the mechanical aspects of locomotory movements leads, as shown above, to two main conclusions: first, the whole of the musculature of a moving animal must be regarded as a single functional unit, any change in the activity of one muscle necessitating changes in all the others; second, the characteristic movements of an animal's limbs bear a fairly close relationship to the mechanical forces against which the limbs are exerting their effort. It may therefore be appropriate to conclude this article by a brief reference to the extent to which purely physiological considerations reinforce or conflict with these conclusions. It is, however, only possible to do so within reasonable limits by raising one or two specific questions and attempting to answer them by reference to particular types of animal. For example, how far is the normal diagonal pattern of limb movement exhibited by the limb of such an animal as a toad dependent on the integrity of the proprioceptor organs located in its own muscles or in those of other limbs? To this question it is possible to give a relatively clear-cut answer (Gray & Lissmann, 1940, 1946). If all the dorsal roots of a limb are severed, the limb muscles lose their tone, and the limb takes no part in supporting the body when the animal is at rest; it can be placed in any posture without eliciting any response. As soon as the animal begins to walk, however, the de-afferentated limb at once takes its proper place in the typical diagonal limb sequence—flexing and extending at the proper time. As soon as the animal ceases to walk, the desensitized limb becomes completely inert. A similar phenomenon is observed if two or even three limbs are de-afferentated. Still further de-afferentation elicits the significant fact that the normal sequence of limb movements persists provided that the afferent and efferent nerves of a

FIG. 6. SUCCESSIVE PICTURES OF MAN RUNNING



Muybridge (1899) by permission of Chapman & Hall

single spinal segment are intact. The site of the intact segment is immaterial—it may supply part of the trunk musculature or part of one of the limbs. The effect is the same provided that the number of intact sensory fibres is approximately the same. It should also be noted that all rhythmical activity is lost if part of the musculature is de-afferentated and the rest de-efferentated; in order to maintain an ambulatory rhythm over the whole of the four limbs it is essential for afferent impulses to enter the cord from a small field of active muscles. The wide range of musculature over which such a segment exerts its proprioceptor influence clearly conforms with the conclusion that the whole of the somatic musculature of a vertebrate must be regarded as a single functional unit in which the activity of each individual part exerts an influence on that of each one of the others. Further, the responses elicited from the de-afferentated limbs of a toad by stimuli applied at various parts of the body are such as might have been foretold from the mechanical considerations discussed earlier in this article. The only obvious function which cannot be induced in a de-afferentated limb by the activity of one or other of the other intact limbs is its ability to maintain a steady prolonged thrust against an external resistance.

So far as the lower vertebrates (fish and amphibia) are concerned, the whole of the facts derived from both physiological and mechanical analysis seem to yield a relatively consistent picture. Considerable care must, however, be exercised before assuming that a similar degree of consistency applies to higher forms. In mammals the earlier work of Sherrington (1892, 1906) and Graham Brown (1912a, 1912b, 1913) seemed to indicate the existence of a locomotory centre within the brain, capable of maintaining rhythmical limb movements in the absence of any afferent proprioceptor impulses; and these authors regarded the role of such impulses as regulatory influences operating on a rhythm initiated intrinsically by the central nervous system. More recent work suggests that the existence of a " locomotor centre " must still be regarded as uncertain.

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THE ROLE OF THE CELL MEMBRANE IN MUSCULAR ACTIVITY

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1 Physical properties of cell membrane
2 Membrane structure
3 Special function of muscle membrane
4 The muscle impulse
5 Properties of the myoneural junction
6 Spontaneous activity at the nerve-muscle junction: quantal release of acetylcholine
7 Nature of the " end-plate reaction "
8 Conclusions
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In the study of excitatory events, the properties of the cell membrane occupy a central position. The surface membrane of a nerve or muscle fibre serves two main functions: (i) it is concerned with excitation and with the rapid spread of activity throughout the cell, (ii) by providing a diffusion barrier to electrolytes, it plays an important part in the preservation of the steady state of the cell and in the maintenance of electrical and chemical potential differences between the interior and the surrounding fluid.

1. Physical Properties of Cell Membrane

There are two main lines of evidence which demonstrate the importance of this surface structure. Electrical measurements have shown that the cell surface is the seat of a resting potential, of 50–100 mv, the interior being charged negatively with respect to the surroundings, and that excitation is accompanied by a momentary reversal of this potential difference (p.d.) (Hodgkin, 1951). Furthermore, the surface layer of a nerve or muscle fibre has a characteristic electric impedance: it offers very high resistance to direct current, and a low impedance to high-frequency alternating current (Cole, 1940; Hodgkin & Rushton, 1946; Katz, 1948, 1950, 1952). It behaves like a thin semi-insulating lipoid membrane of low electric conductance (10^{-3} to 10^{-4} ohm^{-1} per cm.^{2}) and large capacity (1 to 10 μF per cm.^{2}).

With radioactive tracers, the speed of mixing between intracellular and extracellular electrolytes, especially of sodium and potassium, has been studied (Keynes, 1949, 1954; Harris, 1950; Harris & Burn, 1949). These experiments have shown that the exchange of ions between cells and surroundings is very slow. The restraint of the ionic movement must be largely in the cell surface, for labelled Na and K ions which have entered the cell can be seen to diffuse, and migrate electrically, along the interior, with a speed corresponding to that of unrestrained diffusion, and conduction, in an aqueous medium (Hodgkin & Keynes, 1953; Harris, 1954).

2. Membrane Structure

In a qualitative way, the two sets of experiments employing, respectively, electrical and tracer techniques gave the same answer, indicating that there is a special membrane of low and selective ion permeability at the surface of the cell. It has been argued, nevertheless, that the membrane concept rests entirely on indirect evidence and lacks a structural basis, one which can be identified microscopically. This never seemed a very formidable objection, for the measurements of the membrane capacity suggested that the surface structure which one would have to look for was probably no more, and possibly less, than 100 Å units thick (Davson & Danielli, 1943). Such structures have now come within the range of resolution of the electron microscope (see Bennett & Porter, 1953; Draper & Hodge, 1949), and recent electron micrographs by Robertson (1956) have in fact revealed the presence of a discrete surface-membrane complex in skeletal muscle fibres, comprising three layers, altogether 400–700 Å units thick. It is conceivable that one of these layers possesses the characteristic properties, high electric resistance and restricted ionic permeability, which we have been accustomed to ascribe to the cell surface.

3. Special Function of Muscle Membrane

The specialized function of the muscle membrane is to receive the motor impulse, distribute it over the whole length of the muscle fibre, and to pass it on rapidly to the contractile material in the interior of the fibre (Hill, 1950; Sandow, 1952). The mechanism by which this sequence of excitatory events proceeds is not invariably the same in the different skeletal muscle elements. The majority of " voluntary " muscle fibres receive their innervation at one, or a few, localized end-plates —that is, at microscopic focal contact regions between nerve and muscle cells. At these points, a new excitation wave is initiated in the muscle membrane and is rapidly propagated towards the ends of the fibre. There are, however, some slowly contracting " tonic " muscles in the frog in which a different mode of innervation has been discovered: motor nerve-endings are spread out along the length of individual muscle fibres. A simultaneous activation of the whole fibre length is ensured in these cases by the scattered nerve supply, and a muscle cell of this type can dispense with a propagation mechanism of its own. In fact, recent work of Kuffler & Vaughan Williams (1953) and of Burke & Ginsborg (1955) has established that this kind of fibre is unable to conduct action potentials, in contrast with the focally innervated twitch-producing muscle elements.

In spite of these differences, certain common features may be pointed out in the process of activation of " slow " and " fast " muscle fibres. In both, the innervated parts of the cell surface become depolarized by the neuromuscular transmitter (which appears to be acetylcholine in both cases). In slow as well as fast elements, depolarization of the surface membrane invariably precedes, and presumably initiates, contraction in the interior. The principal difference lies in the mechanism by which depolarization is distributed from point to point along the fibre: in the focally innervated elements responsible for the twitch response this requires the propagation of an impulse as an extra step; in the slow fibres, the process has been taken care of by the distributed arrangement of the nerve supply. A situation analogous to that of the slow fibres of the frog may be of wider occurrence: similar properties have been observed in the striated muscles of arthropods (Wiersma,

1941; Fatt & Katz, 1953; Hoyle, 1955), and there is evidence suggesting that mammalian intrafusal muscle elements may behave in the same manner (Hunt & Kuffler, 1951).

4. The Muscle Impulse

The membrane changes which occur during the propagation of the action potential are essentially alike in skeletal muscle and in non-medullated nerve fibres. In both, activity is transmitted along the cell by means of local currents which flow between the resting and excited part of the fibre surface (Hodgkin, 1951). Excitation occurs at every point when the resting potential is suddenly displaced to a lower " threshold " level. In the twitch-fibres of the frog, an action potential is initiated when the p.d. across the membrane is lowered, e.g., by the passage of an outward current pulse, from its normal resting level of about 90 mv to a critical value of about 50 mv (Fatt & Katz, 1951; Nastuk, 1953a; Jenerick & Gerard, 1953). At this point the membrane potential becomes unstable, and the potential change continues automatically, giving rise to a brief spike of about 130 mv amplitude. During the peak of the spike, the p.d. across the membrane is about 40 mv, inside *positive*, of opposite direction to the resting potential. There is evidence that, as in the nerve axon, the muscle spike arises from a characteristic cycle of ionic permeability changes, the rising phase of the action potential being associated with a momentary increase of Na permeability, the falling phase with a slower increase of K permeability. The evidence for this view has been reviewed elsewhere (Hodgkin, 1951). It rests on a great variety of experimental findings: such as (i) the characteristic relation between resting potential and K concentration (Curtis & Cole, 1942; Ling & Gerard, 1950); (ii) the characteristic relation between action potential and Na concentration (Hodgkin & Katz, 1949; Nastuk & Hodgkin, 1950; Desmedt, 1953); (iii) the observation of a large reduction of the membrane resistance during the spike potential (Cole & Curtis, 1939; Katz, 1942; Fatt & Katz, 1951); and (iv) the finding of an increased exchange of external Na for intracellular K during periods of electric activity (Keynes, 1951).

The source of energy for the propagation of an all-or-none impulse is derived from the concentration gradients of Na and K ions across the fibre membrane. The conduction of an impulse involves the entry of a minute amount of Na into the fibre (some 4×10^{-12} equiv. per cm.^{2}) and an equivalent leakage of K into the surrounding fluid. It is interesting that these conclusions have confirmed in essence some shrewd predictions made by Overton more than 50 years ago. Overton (1902) inferred from a series of experiments on frog muscle that extracellular Na is essential for the maintenance of electric excitability, and that a momentary cation exchange across the membrane might be the source of the action currents. In Overton's view, the most formidable problem to be tackled was the question how nerve and muscle cells manage to replenish their ionic store and maintain high concentration gradients against the leakages caused by incessant transmission of signals to or from the central nervous system. Experiments on this problem have been in progress for some years (Steinbach, 1954; Hodgkin & Keynes, 1955) and, in the case of non-medullated axons, have provided good evidence for the existence of an enzymic " pump " which is responsible for continuous extrusion of Na and accumulation of K in the nerve fibre. Hodgkin and Keynes have been able to demonstrate that the outward movement of Na can be effectively stopped by the application of certain metabolic inhibitors (e.g. dinitrophenol, cyanide, azide). Similar experiments have been made on skeletal muscle, so far with negative results (Keynes & Maisel, 1954): it appears, therefore, that the recovery process, whereby ionic concentration and electric potential gradients are maintained across the cell membrane, differs in nerve and muscle fibres. The chemical sources of energy have yet to be identified in either case.

5. Properties of the Myoneural Junction

While the propagation of activity along the " twitch-fibres " of skeletal muscle proceeds in a manner exactly analogous to the nerve impulse, a very different process occurs at the motor end-plate where the nerve impulse is received by the muscle fibre. The work of Dale and his colleagues (Dale, Feldberg & Vogt, 1936; Brown, Dale & Feldberg, 1936) has established that a chemical stimulant, acetylcholine, is released from the nerve terminals and, acting on specific chemo-receptors in the muscle end-plate surface, starts another action potential wave.

The motor end-plate is structurally a highly specialized part of the muscle membrane, thrown into surface grooves in which the terminal branches of the motor axon are embedded. Robertson's (1956) electron micrographs show that there is, at the myoneural junction, not only a close contact between presynaptic and post-synaptic membranes, but a large expansion of the receptive surface of the muscle membrane, brought about by an abundance of tubular folds.

The mechanism of neuromuscular transmission has been studied in recent years with improved electrophysiological techniques in which use has been made of intracellular recording from the end-plate region of the muscle fibre and of micro-application of pharmacodynamic substances to single end-plates. It has been possible in this way to trace some of the intermediate steps in the transmission process, intervening between the arrival of the motor nerve impulse at the terminals and the departure of the muscle impulse from the end-plate.

One of the important features which could thus be investigated with greater accuracy was the " end-plate potential " (e.p.p.), a distinct local depolarization which a nerve impulse sets up in the junctional region of the muscle membrane, and which in turn gives rise to a muscle impulse. The e.p.p. is an essential intermediary between nerve and muscle action potential, and arises directly from the reaction between the transmitter acetylcholine and the surface receptors in the end-plate region. A similar local depolarization can be produced by artificially applied acetylcholine, and both the " natural " and " artificial " potential changes are equally inhibited by curare and enhanced by anticholinesterases (see, for example, Kuffler, 1943; Fatt, 1950; Castillo & Katz, 1955a).

6. Spontaneous Activity at the Nerve-Muscle Junction: Quantal Release of Acetylcholine

The distinctive receptor-properties of the end-plate can be revealed by electric recording, even in the absence of nerve impulses or of artificially applied chemical stimulants. The junctional region of the muscle fibre is subject to a bombardment by minute quantities of acetylcholine which are released spontaneously, at random moments, from the nerve-endings. This results in the appearance of " miniature end-plate potentials ", small local depolarizations of the fibre membrane which are similar to the full-size e.p.p. elicited by a nerve

impulse, but differ from it by their much smaller amplitude and their random occurrence (Fatt & Katz, 1952a).

A detailed study of this spontaneous—and strictly localized—electric activity has led to a number of interesting conclusions (see Fatt & Katz, 1952a; Castillo & Katz, 1954a, 1954b, 1956; Boyd & Martin, 1955). A miniature e.p.p. is due to the release of a packet of acetylcholine from the nerve-endings. The event takes place in the immediate vicinity of the end-plate receptors and involves the synchronous liberation of a large number, possibly some thousands, of acetylcholine molecules. A motor nerve impulse leads to the synchronous discharge of many such quanta of acetylcholine, the normal full-size e.p.p. being made up of a few hundred miniature units.

The nature of this quantum of acetylcholine release presented a puzzling problem, for while it would have been easy to understand that acetylcholine molecules can leak out gradually from their storage place in the nerve-endings, it is more difficult to see what causes them to appear in quanta, in discrete parcels of more or less constant size. The size of this quantum seems to be remarkably independent of the various experimental procedures which have been brought to bear on this phenomenon. Thus, drastic changes in the ionic environment, or in the level of the membrane potential of the nerve-endings, do not alter the quantum appreciably, though the frequency of the discharge can be changed over a more than thousandfold range by these agents. One can say that the statistical chances of any one quantum being released during a given interval of time are extremely sensitive to the experimental conditions and are delicately controlled by the membrane potential of the nerve-endings (Castillo & Katz, 1954b), by the temperature and by the chemical composition of the surroundings (Fatt & Katz, 1952a). On the other hand, the quantity of transmitter which is liberated during each unitary event is relatively fixed (Castillo & Katz, 1956), and it looks as if there are pre-formed parcels of acetylcholine inside the nerve terminals whose sizes are not readily controlled by external influences.

It had been suggested for a long time that the transmitter is synthesized in chemically bound form and stored in intracellular corpuscles. Quite recently, Palade (1954) and Robertson (1956) have observed an accumulation of submicroscopic " vesicles ", of a mean diameter of about 200–300 Å, inside the motor nerve terminals, and these might conceivably be the structural containers of our acetylcholine quantum (see also Bennett (1955); de Robertis & Bennett (1955); and Palay (1954)). That analogous situations may occur more generally, at other " neuro-secretory " terminals, has already been pointed out by Welsh (1955) in an excellent review of this subject.

Though the miniature end-plate potentials are recorded post-synaptically, from the muscle fibre, their frequency gives one a useful indication of the physiological state of the nerve-endings; for example, the rate of the discharge can be controlled electrotonically by passing a suitable steady current through the terminal portion of the motor axon (Castillo & Katz, 1954b). In these experiments it was found that transmission of electrotonic potentials proceeds up to the ends of the motor axon, but fails completely to spread across the myoneural synapse. This provided interesting evidence for the inadequacy, indeed the non-existence, of electric transmission at this junction: a conclusion which is complementary to our concepts of a special chemical transmitter.

It was of some importance to localize the position of the acetylcholine receptors in the motor end-plate membrane. It had been assumed that the transmitter substance, after its release from the axon terminal, collides and reacts with special molecules in the external surface of the post-synaptic membrane. An alternative view, however, had been put forward according to which the acetylcholine reaction takes place " intracellularly " (Nachmansohn, 1946). This hypothesis has been subjected to a direct test by applying small quantities of acetylcholine extracellularly as well as intracellularly (Castillo & Katz, 1955a). A micropipette was filled with acetylcholine, and the release of the substance from its tip was controlled electrophoretically. The tip of the pipette was applied close to a motor end-plate, and in successive trials was alternately placed on the outside or made to penetrate the muscle membrane. The result was unequivocal: only external application depolarized the end-plate and initiated a muscle impulse; intracellular release of acetylcholine was ineffective.

7. Nature of the " End-Plate Reaction "

A series of experiments has been made to throw some light on the process by which acetylcholine alters the potential of the end-plate membrane. It has become clear that, as a result of the transmitter action, ionic current of a few microamperes is allowed to traverse the end-plate membrane momentarily, and that the quantity of charge which is transferred in this way greatly exceeds the charge attached to the available acetylcholine ions (Fatt & Katz, 1951, 1952b; Nastuk, 1953b; Castillo & Katz, 1956). The current must be carried predominantly by other ions, and it appears that acetylcholine primarily causes an increase of the membrane permeability to ions which are present in abundance in the environment.

A permeability change of a similar kind occurs during the electric excitation of nerve or muscle fibres. In this case, the effect of the electric stimulus is to depolarize the membrane partly; this causes a specific rise of permeability to Na which leads to a regenerative reinforcement of the initial potential change (Hodgkin, 1951). The decisive evidence for this view was obtained when it could be shown that the membrane potential, during electric excitation, moves towards a new level which is uniquely determined by the electro-chemical equilibrium for Na ions (Hodgkin & Huxley, 1952).

The e.p.p. arises, however, by a rather different mechanism. In the first place, it is started by a chemical reaction, not by an electric current. Secondly, the e.p.p. moves towards an equilibrium level which is much lower than the " Na-potential " (10–20 mv inside negative as compared with 40–50 mv inside positive). This has been shown for twitch-fibres as well as for the slow fibres of the frog (Castillo & Katz, 1954c; Burke & Ginsborg, 1955). The result can be explained if acetylcholine produces a general increase of ionic permeability, one in which both Na and K, and perhaps all free ions, are equally and simultaneously involved. The transmitter may be visualized, somewhat crudely, as producing a transient local puncture of the end-plate membrane; this short-circuits the surrounding muscle membrane and causes its potential to drop to the level at which electric excitation occurs and a muscle impulse is set up.

It has been possible to test certain predictions of this " short-circuit " hypothesis. Thus, it was confirmed that the transmitter lowers the resistance of the end-plate membrane and produces an extra leakage path, in parallel with that occurring

during direct electric excitation of the muscle fibre (Castillo & Katz, 1954c).

Furthermore, acetylcholine was found to increase the electric conductance of the end-plate membrane even when all the Na in the surrounding medium has been replaced by K (in the form of an isotonic solution of K_2SO_4); this indicates that the ionic permeability change is not restricted to Na alone (Castillo & Katz, 1955b).

8. Conclusions

The function of the muscle membrane is to receive the stimulus from the nerve and transmit it to the contractile substance. How excitation is passed on from the surface to the interior of the muscle fibre has so far eluded clarification. But the mechanisms of reception of the signal and of its distribution along the fibre surface are now fairly well understood. A special situation exists at the myoneural junction where a large post-synaptic cell is triggered into activity by an impulse arriving in small presynaptic axon branches. Direct local-circuit transmission comes to a halt at this point and is replaced by chemical transmitter action. Acetylcholine is released, in discrete quantal parcels, from the nerve-endings and, acting on receptors in the external surface of the end-plate membrane, causes a large increase of permeability to Na, K and possibly other free ions. This gives rise to local currents of sufficient intensity to depolarize and excite the surrounding area of the muscle fibre and so initiates a new propagating impulse.

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NERVOUS GRADATION OF MUSCULAR CONTRACTION

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When a certain movement is to be made, the motor centres have to bring into action the necessary muscles in the correct sequence and also—and this is the problem with which we are concerned here—to ensure that at each instant the strength of contraction in each muscle is accurately graded to its task, for example, to support the hind-quarters of a cat, or to hold up 5 lb. (2.3 kg.), or to slew the eyeballs through 40°. The general principle on which the nervous system does this is clear enough. It employs sensory receptors to report how much of the object is accomplished and, in the light of this information, or " feedback ", it modifies the rate of motor discharge. As a man stretches out his arm to pick up a pencil, information on the position of his fingers is fed back to the brain by his eyes and used to steer them. If he shuts his eyes he will probably miss and be reduced to groping. It might be possible to reach the pencil using only visual feedback, but in practice several other sorts of information are used, which do not necessarily reach consciousness: for example, positional information from the joints of the arm, touch information from the fingers and information from muscle sense organs—particularly the muscle spindles, whose job it is apparently to monitor the performance of the muscles themselves. If these sources are cut off, the arm becomes almost unmanageable under visual control alone, and the task of getting the pencil, if accomplished, would be very slowly and imperfectly carried out. It is with the muscle spindles that we are largely concerned here, for they seem to have a good deal of the responsibility for seeing that the muscle does just what is asked of it.

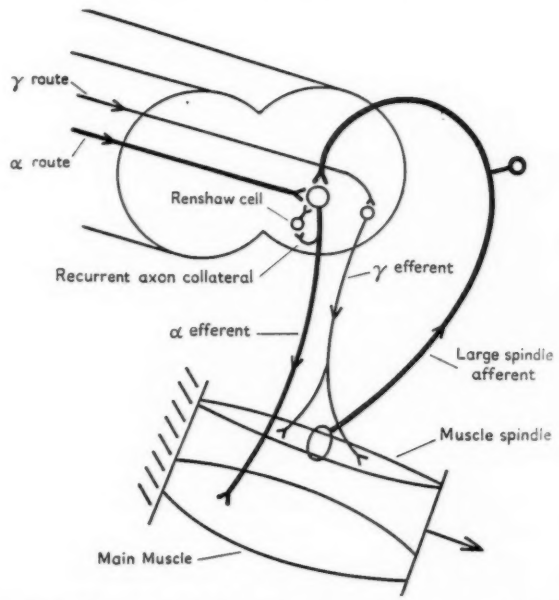
1. Postural Type of Contraction: the " Length Servo "

The muscle spindles lie among the main muscle fibres and share their attachments, so that they are extended when the muscle lengthens and relaxed when it shortens (fig. 1) (Sherrington, 1900). The reflex connexions are such that impulses set up by stretching the spindles excite the muscle's own motoneurones. Thus extension of the muscle results in an augmented contraction which tends to resist the extension (fig. 1). This mechanism is the " stretch-reflex " (Liddell & Sherrington, 1924) and we see it has the properties of a " length servo ", i.e., a self-regulating closed-loop mechanism using negative feedback from the spindles to maintain a constant muscle length.

The advantages of a length servo over a simple straight-through system (in which posture is maintained by a steady stream of motor impulses to the muscle without sensory feedback) is that it automatically compensates for changes in load or for fatigue (see Eldred, Granit & Merton (1953) and the paper by Merton in this number of the Bulletin, p. 219).

Evidence that during a postural type of contraction the muscle really is under this kind of servo-control first came from investigation of the " silent period ". If a muscle is

FIG. 1. DIAGRAM OF THE STRETCH-REFLEX AND RELATED MECHANISMS



The muscle spindle lies in parallel with the main muscle and its fast conducting afferent is in synaptic connexion with the large α motoneurone supplying the main muscle fibres. A slow conducting γ motor efferent (thin line) supplies the contractile poles of the spindle and thus can alter the bias on the spindle sensory ending. The muscle can be made to contract either by impulses from higher centres exciting the α motoneurone direct (the α route) or by impulses in the γ efferents (the γ route) which activate the muscle indirectly via the stretch-reflex arc (the " follow-up " servo). A subsidiary feedback loop via the recurrent axon collateral and an inhibitory Renshaw interneurone may be concerned in stabilizing the response of the α motoneurone to its excitatory input.

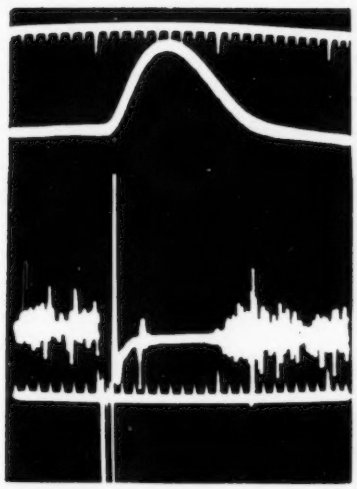
made to twitch (e.g., by an electric shock) during a steady contraction, the electromyogram is silent during the superimposed twitch. The interpretation is that muscle shortening occurs during the twitch, which slows the discharge from the spindles (as demonstrated by Matthews (1933)) and thus withdraws excitation from the motoneurones. A steady voluntary effort in the human subject also seems to employ the length servo, for a silent period is easily demonstrated during voluntary effort (fig. 2), and analysis shows that it has the mechanism of a length servo.

Conversely, stretching a muscle during voluntary effort calls forth an opposing contraction (Hammond, 1954) which, from its brief and constant latency, is clearly a stretch reflex. In these experiments the subject pulled steadily on a wristlet, using his elbow flexors. Without warning the wristlet was pulled away at a constant velocity and the tension in the connecting tape recorded. The subject was instructed to resist the impending pull. Typical responses are shown in Pl. V, A. The onset of extension is marked by a sharp rise in tape tension as the suddenly applied velocity accelerates the effective mass of the limb. Subsequently there is a transient oscillation in tension, due to a speed fluctuation of the driving motor in response to the sudden loading. During this period the mean level of tension is increased above initial tension by the passive force needed to extend the muscles at a constant rate. After a latency varying from subject to subject, but lying between 60 and 80 msec., the tension in the tape exhibits an abrupt rise. Electromyograms from surface electrodes over biceps show an outburst of activity starting slightly before the tension rise. Earlier in the electromyogram record, some 15–20 msec. after the start of the pull, is a diphasic wave representing the tendon jerk. (As observed in cats by Denny-Brown (1928), the tendon jerk elicited when a muscle is already contracting does not cause a detectable mechanical twitch, probably because the diphasic wave only involves fibres which were about to discharge anyway, so that the mean rate of discharge is little affected and no significant change in tension occurs.)

An experiment to determine the reaction time to a sharp tap on the wristlet yields the records in Pl. V, B. Here the subject is instructed to pull the tape as hard as possible when his wrist is tapped. The diphasic wave in the electromyogram occurs as consistently as before, but the delay between tap and rise in tape tension is at once longer and less consistent than was the reaction to an extension. Forcible extension of the flexor muscles thus produces an almost maximal response in roughly half the time taken to react to a tap. The consistency and short latency of the muscular response to an extension lead to the conclusion that here a purely spinal reflex is at work, whereas the response to a tap involves pathways in the brain. It is clear that the shorter latency in response to extension, compared with the latency to a tap, stems from the sustained stretching of the muscle and that the response is that of a stretch reflex or, in other words, a positional servomechanism which resists externally imposed postural changes.

It was observed that the rate of rise of tension during the stretch reflex was the same as in a maximal voluntary pull against a stop and was therefore probably as fast as the muscle could achieve (Merton, 1954).

FIG. 2. THE SILENT PERIOD IN THE ADDUCTOR POLLICIS MUSCLE OF THE HUMAN HAND



The subject makes a steady voluntary contraction of 1 kg., and the associated action potentials (the electromyogram) are recorded. When the muscle is excited by stimulating the motor nerve with an electric shock, the electromyogram is silent during the superimposed twitch. This is because impulses from the muscle spindles cease during the twitch (see fig. 1) and thus excitation is withdrawn from the α motoneurones (Merton, 1951).

The record consists of ten traces superimposed, to demonstrate the great regularity of the phenomena.
Twitch tension : roughly 1 kg.
Action potentials recorded with a needle electrode
Time markers (top and bottom) : 10 msec. and 100 msec.

2. Muscle Shortening and Lengthening: the " Follow-Up Length Servo "

The stretch reflex so far described is excellent for holding a fixed posture, but such inflexible behaviour would soon be embarrassing to an animal. The way round this difficulty was first seen by Rossi (1927) who remembered that the poles of muscle spindles are contractile and receive a motor supply (called, from its fibre size group, the γ efferents, the main muscle being supplied with larger α efferents). The sensory portion of the spindle lies between the contractile poles, hence shortening of the poles will have the effect of " biasing " the sensory ending so that the same rate of discharge will occur at a shorter muscle length (Matthews, 1933; Leksell, 1945; Kuffler & Hunt, 1952). Consider a muscle in postural contraction at a certain length. If motor discharge in the γ efferents causes shortening of the spindle poles, this will stretch the sensory portion, so that the stretch reflex will be activated just as if the muscle itself had been stretched. The muscle will shorten reflexly until the increased rate of spindle discharge is offset, and that will be (to a first approximation) when the muscle has shortened to the same extent as the spindles. Hence the complete stretch reflex mechanism, including the γ efferents, constitutes a " follow-up servo ", the muscle length tending to follow changes in spindle length. The spindle endings themselves do not in fact record length, but the differences in length between muscle and spindle ; they are misalinement detectors.

To establish the theory, Eldred *et al.* (1953) observed the contraction of the gastrocnemius-soleus muscle of a cat and at the same time listened to the discharge in one of its muscle

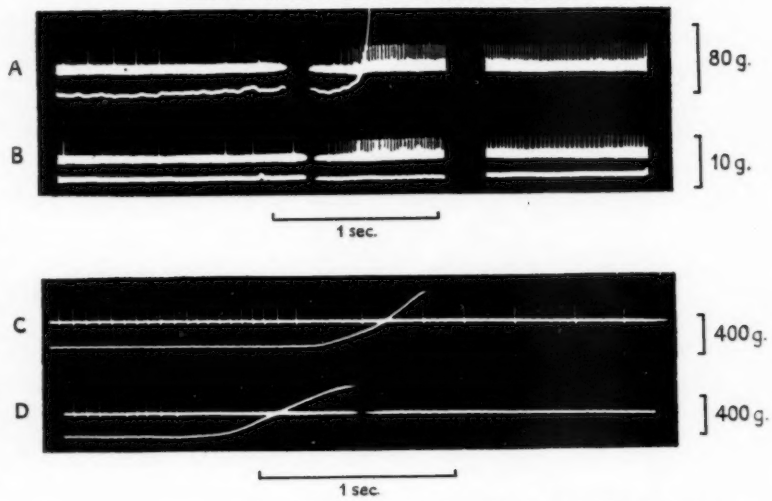
spindles. To do this it was necessary to cut only a small strand of a single dorsal root, so that the innervation of the muscle was not significantly reduced. As was known from earlier work, various manoeuvres—such as twisting the ear—which caused contraction of the muscle also resulted in great acceleration of the spindle discharge (fig. 3A). This implies a rapid γ discharge to the spindles, making them contract faster than the main muscle, for a spindle " in parallel " with the main muscle stops discharging when shortening occurs. Clearly this behaviour is just what is to be expected if the γ efferents are operating a follow-up servo. Finally the servo-loop was opened by severing all the relevant dorsal roots. Afterwards no contractions were obtained, but acceleration of the spindle was observed just as before (fig. 3B).

3. The Direct Pathway

While the existence of a follow-up length servo seems to be as well established as most concepts in this field, it is perfectly clear from many experiments on de-afferented limbs (in which the servo is necessarily out of action) that movements can often occur without it. Indeed the delay entailed in conduction time down the small γ efferents and up the spindle afferents (which could amount to as much as 50 msec. in the case of the hand) suggests that sudden movements might take a short cut direct to the muscle through the α efferents. Granit, Holmgren & Merton (1955) showed that, by cooling the anterior lobe of the cerebellum (or by other interference in that region), it was possible to re-route reflex contractions so that exactly the same reflex (e.g., a contraction of gastrocnemius from pinching the ear) which initially used the follow-up servo now used the direct route. This was evidenced by a reversal of spindle behaviour; the sample spindle which was under observation now slowed during contraction instead of greatly accelerating (fig. 3C). Cutting the dorsal roots had no influence on the reflex contraction, whereas previously it had abolished it (fig. 3D). These results show that the γ efferents are no longer activated during the reflex and that the servo-loop is not in use, as it can be opened with impunity. However, γ paralysis is not the only effect, for, if it were, the reflex contraction should be impossible to elicit (as it was in the previous instance after de-afferentation). As the reflex excitability did not change consistently, the excitation withdrawn from the γ motoneurones must be redirected to the α route. The tentative conclusion is that the cerebellum (and associated structures) determine whether the length servo or the straight-through mechanism is to be used in contraction. This fits in with and extends the current notion that the cerebral cortex decides *what* is to be done and the cerebellum has the job of arranging *how* it is to be carried out.

On the human subject it has also been found that a given contraction can be carried out in two ways, depending on the subject's intentions (Hammond, 1956). The responses in Pl. V, A were obtained with the subject attempting to resist a pull; those in Pl. V, C show the effect of instructing the subject to let go when pulled. It is apparent that prior instruction exerts a profound influence on the vigour of a response. The tension responses to the instruction " let go " are very much more erratic in shape than are the responses to the instruction " resist "; this is in accord with a reflex mechanism which favours maintenance of posture as opposed to acquiescence in postural changes, but which the subject can be trained to overcome. The point of divergence of the tension rise as between " resist " and " let-go " instructions occurs where the " resist " responses first begin to rise. Since the abrupt tension rise in a " resist " response is due to a stretch reflex, it follows from the evidence in Pl. V, A and C that the subject can interfere with the stretch reflex to the extent of suppressing it altogether. If the executive organ has control of reflex activity in this way, then its scope is clearly extended to situations where the presence of a stretch reflex might be a disadvantage to the performance of a controlled movement.

FIG. 3. MUSCULAR CONTRACTIONS INITIATED VIA THE FOLLOW-UP SERVO AND VIA THE DIRECT ROUTE



Adapted from fig. 10 of Eldred, Granit & Merton (1953) and fig. 9 of Granit Holmgren & Merton (1955) by permission of Journal of Physiology

In these experiments on decerebrate cats, contraction of the ankle extensors is induced reflexly by moving the head up and down. Muscle tension is recorded (continuous line) together with the spike discharge from a single muscle spindle. The interruption of the traces in A and B signals the time of head movement, but was late in fig. D.

A, B : decerebration by intercollicular section

A : muscle contraction accompanied by great acceleration of spindle discharge, indicating that the follow-up servo (γ route) is activated

B : this is confirmed after cutting the dorsal roots, when spindle acceleration occurs as before, but there is no contraction visible even with the greater sensitivity of the myograph

C, D : another cat decerebrated by tying the basilar artery. This also kills the anterior lobe of the cerebellum and favours the direct (α route) of excitation

C : contraction is not accompanied by spindle acceleration

D : after cutting dorsal roots contraction occurs as before. The α motoneurones are no longer dependent on spindle drive

RESPONSES TO SUDDEN STRETCHING OF THE FOREARM FLEXOR MUSCLES IN THE HUMAN SUBJECT

(FIGS. A—D)

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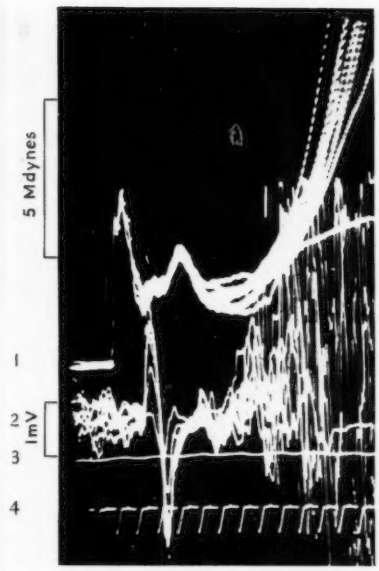
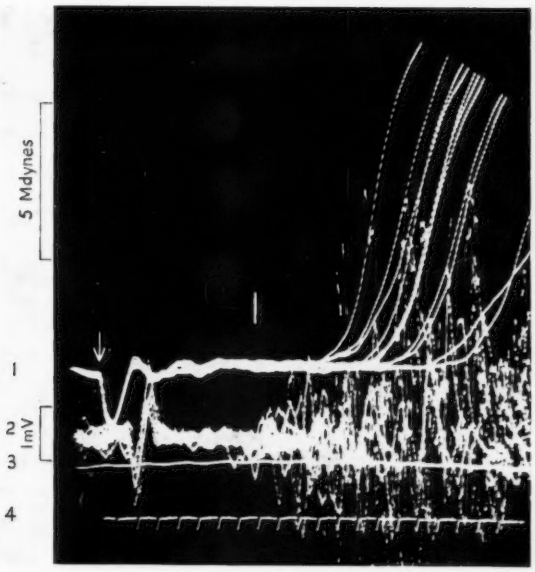
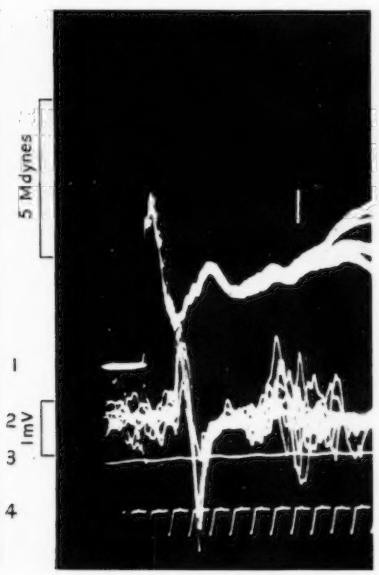


A: subject instructed to resist impending pull. Ten superimposed records of pulls at 40 cm. sec. at the wrist



B: ten responses to taps on the front of the wrist delivered at the instant marked with an arrow. Subject instructed to pull hard as soon as he feels the tap. Electrode connexions reversed compared with A



C: subject instructed to let go when pulled. Conditions otherwise as in A

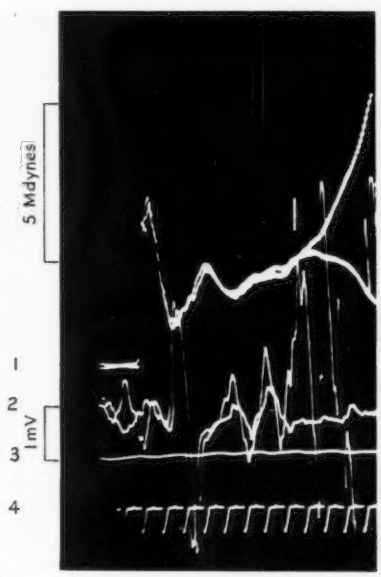
In each figure the records in descending order are:

1: tension in the steel tape pulling on the wristlet. The initial tension is always 3 Mdynes (3.06 kg.)

2: electromyographic potentials from surface electrodes over biceps

3: zero tension base line

4: 10-msec. time markers



D: comparison of one "resist" and one "let-go" response. Conditions as in A and C

On each figure a vertical white bar above the tension trace marks the average time of the abrupt tension rise in the stretch reflex.

4. The Renshaw Feedback Loop: the Tendon Jerk

There is now evidence that, even when the α route is in use, motoneurone discharge is subject to servo-control. Renshaw (1941) found that, when an antidromic volley was backfired into part of a motoneurone pool, the other unexcited motoneurones were inhibited. The mechanism has been elegantly analysed by Eccles, Fatt & Koketsu (1954) using Eccles' celebrated technique of recording from inside the motoneurone with a micro-electrode. Branches (" collaterals ") from the motor axons run back into the ventral horn and excite internuncial " Renshaw " cells which in turn inhibit the neighbouring motoneurones. There are various complicating factors that make it far from clear whether it is fair to regard this as merely a negative feedback circuit stabilizing the response of the motoneurone pool in the same way that voltage feedback stabilizes an amplifier, but the experiments of Holmgren & Merton (1954), who used a natural reflex discharge and studied the effect of varying the reflex tension and the size of the antidromic volley, showed that it could behave as if it were such. If this function is definitely established it will be of great interest, as recurrent collaterals are found throughout the nervous system.

Of particular relevance to experiments in muscle excitation is the existence of an appreciable delay (some 2–5 msec.) around the Renshaw loop. This implies that a sufficiently brief input (e.g. a synchronous nerve volley) will discover the sensitivity or " gain " of the loop as it would be without feedback, because it will go through before feedback can act. A most important question to be answered is whether the tendon jerk (or the monosynaptic reflex, which is equivalent) is to be identified with this " unfedback " response, and it must be admitted that many difficulties might be resolved if it were. Granit (1955) has well described the inconsistencies that arise when the state of excitability of motoneurones is tested by eliciting a reflex contraction, or by a tendon jerk or monosynaptic reflex. This discrepancy is also obvious clinically, although little insisted on; for instance, although resistance to passive movement (an index of the stretch reflex) and increase of tendon jerks go together in spastic states, the reverse is so in cerebellar disease where we may find absolutely flaccid limbs with brisk jerks.

A clear distinction between jerk and stretch reflex was also apparent in the experiments on the human stretch reflex with " let-go " and " resist " instructions described above. It was always observed that the diphasic wave was unaffected by the instructions even when the changes in stretch reflex were most complete (Pl. V, D).

The human evidence thus certainly shows that the tendon jerk and the stretch reflex must be considered separately. The reason for the importance of the tendon jerk in medicine may be that it reveals changes in the motoneurones which in ordinary activity are compensated by feedback. If this hypothesis is correct, the status of the tendon jerk is reduced to an accidental overload condition of a nervous pathway.

5. Accuracy of Muscular Control

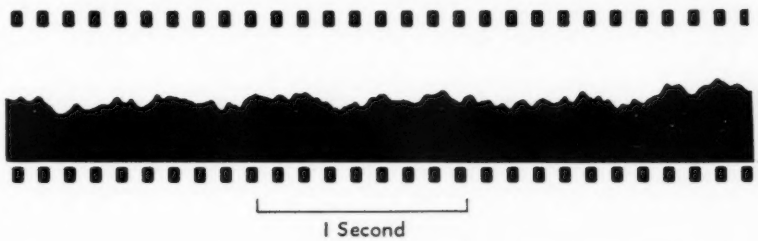
Another approach which yields information about the mechanism of muscular control is to measure the accuracy with which a muscular effort can be made. The equipment used was designed for work on manual tracking (Sutton, 1956), but in the experiments to be discussed the operator had merely to press on a joystick (using his wrist muscles) with a fixed force of, say, 5 lb. (2.3 kg.). A voltage proportional to this force was generated and used to deflect the spot on a cathode-ray tube facing the subject, whose task it was to hold the spot at the 5-lb. mark. The error could either be recorded on film for analysis (fig. 4) or squared and integrated by electrical means at once.

When the demanded force was altered in successive experiments the relative accuracy (measured as the coefficient of variation) remained the same. This means that the sensory feedback mechanisms used to carry out the task treat as of equal significance errors that are a constant fraction of the force exerted, as in Weber's law. Merton (1951) showed that the change in tension needed to produce a silent period was similarly related to the initial force.

Frequency analysis of the error record (fig. 5) shows that the greater part of the error is at low frequencies with a small peak around 9 cyc./sec., as noted by previous workers (e.g. Travis & Hunter, 1931; Sollenberger, 1937; Redfearn & Halliday, unpublished observation). Interest has centred on the 9 cyc./sec. peak because it is felt that, whereas the large activity at lower frequencies represents the total of the tracking performance of all the feedback loops involved, the 9 cyc./sec. peak may be due to slight oscillation of one particular loop, e.g. the length servo-loop. If this were so, any interference with the loop might be expected to modify the 9 cyc./sec. peak. Preliminary experiments have shown that moderate cooling of the forearm (in water at 15° C. for 20 min.) profoundly decreases 9 cyc./sec. tremor, which suggests that the 9 cyc./sec. peak is more likely to be due to oscillation in the muscle servo than, for instance, to modulation of descending activity by the α rhythm.

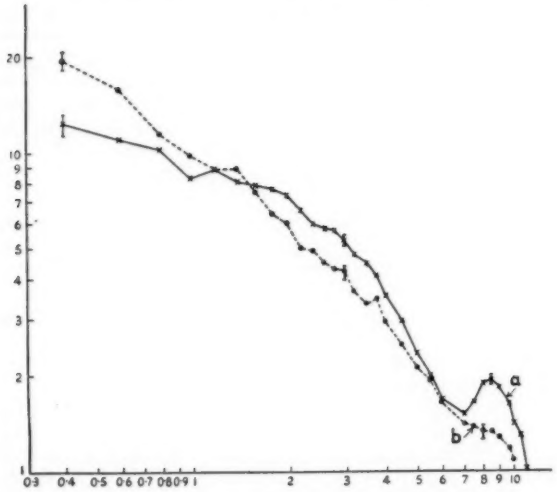
With this experiment in mind it was interesting to find that the 9 cyc./sec. peak could also be reduced by withdrawing visual information during the task. At the end of a 30-second run with normal visual information in the above experiment, the subject was asked to shut his eyes and continue the task with proprioceptive information only, for an equal time. The great reduction in the 9 cyc./sec. peak is shown in fig. 5, and the effect has been fully substantiated in numerous

FIG. 4. RECORD OF TREMOR IN A VOLUNTARY TASK



The subject is attempting to exert a pressure of 5 lb. (2.3 kg.) on a joystick. His inaccuracies, which are shown in the record, are only a few per cent of the total force. The 9 cyc./sec. component of tremor (shown more clearly in the harmonic analysis in fig. 5) can be distinguished here.

FIG. 5. FREQUENCY ANALYSIS OF THE ERRORS IN MAKING A VOLUNTARY EFFORT



Abscissae: frequency (cyc./sec.) on a logarithmic scale

Ordinates: relative amount of error at each frequency expressed as error, in arbitrary force units, per unit bandwidth on a logarithmic scale

The subjects were attempting to maintain a constant force of 5 lb. (2.3 kg.). Each point is the mean of 16 experiments. The vertical bars give the standard deviation of the 16 observations about the mean.

With the eyes open (curve (a)) there is a peak at 8.5 cyc./sec., which is absent when the eyes are closed (curve (b)).

experiments on four subjects. (Similar results were obtained when the subject kept his eyes open but the cathode-ray tube spot was extinguished.)

In themselves the eyes-closed experiments might suggest that 9 cyc./sec. tremor was a modulation imposed by the highest centres but, if the view that 9 cyc./sec. tremor is a resonance in the muscle servo-loop is to be retained, it must be concluded that withdrawing visual feedback reduces 9 cyc./sec. tremor by altering the parameters of the muscle servo-loop; not that such a possibility causes surprise, in view of what is now known of the α and γ modes of contraction.

6. Muscle Spindles in Eye Muscles

The recent discovery of typical muscle spindles in the eye muscles of man and some other animals (see Cooper, Daniel & Whitteridge, 1955) is not least important in furnishing proof that muscle spindles are not concerned in position sense but are, in Granit's phrase, the private measuring instruments of the muscle's servo-mechanisms. As shown by Helmholtz (1867), there is no position sense in the eye muscles; passive movements of the eyeball are unappreciated, and the resulting shifts of the image on the retina are interpreted precisely as if the eye had stood still and the external world had moved. The eyes are directed by judgement (based on past visual experience) of the amount of effort needed to move them. If the relationship between effort and resultant movement is grossly disturbed, for example by curare, the position of external objects is misjudged and eye movements are accompanied by apparent movement of the external world, as one of us (P.M.) has recently confirmed.

Thus none of the extremely numerous proprioceptive endings in the eye muscles have access to consciousness, and experiments recently begun are confirming the old view that the same is true of the tongue and limb muscles. Sherrington (1918) insisted that the sensory endings in eye muscles subserved position sense, but that must have been chiefly because he could not think of anything else for them to do. The function of the muscle spindles, as at present comprehended, is to ensure by feedback action that the contraction is accurately related to the effort made.

In cerebellar ataxy the patient is absolutely unable to make his muscles do as he wishes. Granit *et al.* (1955) suggest that this is because the muscle spindles are paralysed, depriving him of his muscle servo. No loss of sensation whatever occurs in cerebellar disease except that the judgement of weights held in the hand is impaired (Holmes, 1917). This would be unintelligible if the spindles were position indicators, but if they serve to make accurate the sense of effort, it is easy to see why weights should be misjudged. Thus, although messages from muscle spindles do not reach consciousness, the spindle system may be involved in false sensory judgements—for example, apparent movement of objects in the visual field after curare, or erroneous estimation of weights in cerebellar disease.

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PROBLEMS OF MUSCULAR FATIGUE

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1 Site of muscular fatigue
2 Nature of fatigue
3 Fatigue of skilled movements
References

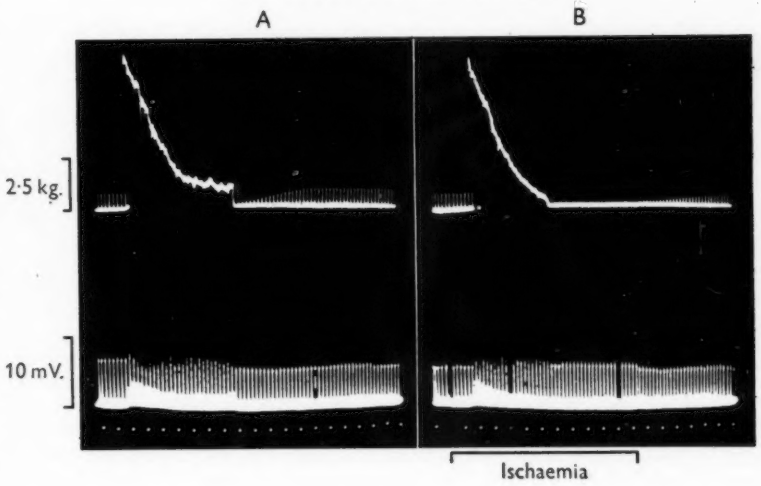
1. Site of Muscular Fatigue

When we indulge in severe exercise, such as weight-lifting or squeezing a dynamometer, strength very soon begins to fail. Anyone who possesses a sphygmomanometer and an open mind can readily convince himself that the site of this fatigue is in the muscles themselves. It is only necessary to perform some act with the hand that leads to fatigue and, at the moment of ceasing, to arrest the circulation in that arm. It will then be found that there is no recovery of strength until the blood flow to the muscles is restored. If fatigue were a phenomenon of the central nervous system, it seems most improbable that recovery should be delayed by occluding the circulation to the arm.

In the past, however, many workers have resisted this conclusion, probably because animal experiments lead to a different expectation. The literature is bedevilled with statements that muscles completely fatigued by voluntary effort can be made to contract by electrical stimulation of their nerves. Reid (1928) even made such an observation in an experiment on an ischaemic arm (like that described above) after time had been allowed for recovery of the central nervous pathways. The simplest explanation of these results (if they were true) would be that not all the motor units were at the command of the will, so that in a voluntary contraction some would escape fatigue. Reid proposed a different hypothesis, namely that inhibitory impulses were set up in the muscles to prevent further voluntary contraction and to protect the muscles from the supposedly dangerous effects of complete fatigue.

In a previous paper (Merton, 1954a) I showed, by simplifying the situation and using only one muscle (the adductor of the thumb) and special apparatus for recording tension, that it was never possible to make the muscle contract by electrical stimulation after it had become fatigued by voluntary effort. Two records from these experiments are shown in fig. 1. Throughout both records the state of the adductor was tested every few seconds by a maximal motor shock to the ulnar nerve. With the muscle relaxed at the start, each volley causes a twitch of about 1 kg. tension and an action potential. The subject then makes a maximal voluntary contraction, exerting a tension of about 7 kg. at the start, which weakens from fatigue over the next two minutes. In fig. 1A, after an initial linear fall, the fatigue curve tends to flatten out. At the end of the voluntary contraction when the subject relaxes, the twitch due to the volley is seen to be very small (although it recovers rapidly), showing that fatigue involves a failure of the muscle to respond to motor impulses. In fig. 1B is a record of a similar experiment in which the circulation to the hand was arrested before the start of the voluntary effort. The fatigue curve now runs straight downwards, suggesting that the previous flattening out was due to partial recovery of blood flow, owing to the fall in intramuscular pressure. Afterwards, no twitch is visible until recovery begins at the time when the circulation was restored. Since the twitch is absent during fatigue there is, at least in this instance, no evidence of inhibition during fatigue, or of a fraction of the muscle not accessible to the will. It is strongly indicated that fatigue is peripheral and is due to failure of the muscle to contract when motor impulses reach it. The experiment also tells us that the failure of the muscle to respond is not due to blockage of impulses at the neuromuscular junction, for the records show that there is

FIG. 1. FATIGUE OF VOLUNTARY CONTRACTION OF THE HUMAN ADDUCTOR POLLICIS MUSCLE



Upper trace: a series of single twitches (evoked by maximal motor nerve shocks) preceding and following a maximal voluntary contraction

Lower trace: the corresponding action potentials. Only the first phase photographed, as described in Merton (1954a)

Time marker: dots at ½-min. intervals

A: record obtained with circulation intact throughout

B: record obtained with circulation occluded for the period indicated

The records show that in the fatigued state the muscle does not respond to a motor volley, and that it does not recover from this condition so long as the blood supply is cut off; both of which facts lead to the conclusion that fatigue is peripheral, in the muscle itself.

no significant falling off in the voltage of the muscle action potentials.

2. Nature of Fatigue

It might be thought—once the motor pathway from the cortex to the propagated muscle action potential is exonerated from responsibility for muscular fatigue—that the problem might without further ado be handed to the biochemist to discover the relevant chemical changes in a fatigued muscle; but this would be premature, for interest has recently centred on the next link in the chain, namely the mechanism by which the action potential sets off the contractile material in the muscle fibre. It has been known since the time of Bernstein (cited by Lucas, 1909) that the wave of contraction passes over the muscle at the same velocity as the propagated action potential. This naturally led to the view that the action potential caused the contraction, but until recently there was little indication which part of the action potential was involved. It is now suspected that the size of the contraction is related to the size of the negative after-potential of the spike (e.g. Hagiwara & Watanabe, 1955). Whether this is so or not, it is perfectly clear that the relationship between action potential and contractile mechanism is graded; even in healthy human muscle, without any question of drugs or fatigue, it can be arranged that two almost identical action potentials give rise to twitches differing by as much as 30% in tension, merely by altering the degree of synchrony of the exciting volley (Merton, 1954b). In the rare disease familial periodic paralysis, the muscle action potential (which is of abnormal form) may be grossly inefficient, giving twitches only one-fifth of the size to be expected from its voltage and from the tetanic strength of the muscle (Merton, unpublished). Thus it is quite wrong to think of the action potential as " triggering " a fixed all-or-none contraction of the muscle fibre. The size of contraction obtained probably depends both on the precise form of the action potential and on the state, as regards fatigue, and so on, of the contractile substance itself.

During fatigue the shape of the recorded action potential is by no means constant, which raises the question whether changes in the voluntary tension exerted may not be due partly to changes in the coupling between action potential and contractile substance. To investigate this, it may be possible by sufficiently brutal electrical stimulation to activate the contractile part directly, even if the action potential is no longer able to do so. Indeed previous experiments of the smoked-drum type, which claimed to demonstrate neuromuscular block by alternate " direct " and " indirect " stimulation of nerve-muscle preparations, may have been partly, or wholly, concerned with the action potential-contraction coupling rather than with the neuromuscular junction (e.g. Brown & Burns, 1949). These experiments often used " galvanic " stimuli (interrupted direct current) for the direct excitation, but it is now known to be more effective to employ alternating current of 200–5,000 cyc./sec. applied transversely to the fibres (Katz & Lou, 1947; Sten-Knudsen, 1954). In man, I found that currents similar to those used by Sten-Knudsen (20 mA/cm.^{2} between two plate electrodes) were unable to cause any contraction of a fully fatigued muscle in the hand, although beforehand a much weaker current gave a large contraction. Thus, to date, there is no evidence that fatigue is due to failure of the action potential-contraction coupling. Larger currents would be desirable but were not used because of pain and the risk of burning, which already occurs if electrode contact is uneven. Fortunately this is a problem where one may hope to get reliable results on animals.

3. Fatigue of Skilled Movements

The foregoing observations on muscular fatigue are clearly of significance to those whose job involves heavy muscular work, but it might be doubted whether failure of the muscles themselves has much to do with the fatigue observed in such light and highly skilled tasks as writing, for it does not seem to need much force to move a pen. When the matter came to be tackled experimentally, unexpected results were obtained. The method was to write with the circulation occluded by a blood-pressure cuff around the upper arm. All goes well for about 1 min. 30 sec., after which difficulties rapidly develop, and in another 30 sec. it has become extremely hard to write at all. While the possibility of being misled is ever present in such experiments, I believe the inability to write is to be attributed to muscular fatigue because (i) some movements, e.g. adduction of the index, have obviously become weak; (ii) no recovery occurs if the subject rests with the cuff on; (iii) there is no interference with writing whatsoever from mere occlusion for periods up to 15 min. Two minutes is a very short time for even ischaemic fatigue to develop (cf. fig. 1), and this indicates that some muscles must be working hard even in ordinary writing. In another type of experiment the subject wrote for 2 min. with free circulation, after which the cuff was applied. Whether he continued writing at once, or took a 2-min. rest first, he was able to continue for only some 30 sec. without severe difficulty. Hence after 2 min. of solid writing there may be very little reserve in some of the relevant muscles, which reinforces the previous conclusions. Four subjects gave concordant figures.

Very similar results were obtained with an expert violinist and with piano-playing. The violinist could play exercises at roughly four notes per second for, on the average, 50 sec. with his left arm ischaemic. The pianist lasted about the same time in fast playing. When the muscular reserve was estimated after 2 min. of playing it was again found to be small in both players.

A second point that emerges is that muscular fatigue makes little difference to performance until the muscles are almost too weak to make the necessary movements. This is of interest in connexion with the theory of the servo-control of muscular movement (see the paper by Hammond, Merton & Sutton in this number of the Bulletin, p. 214). The results, in fact, were unexpectedly good; it was thought that the proprioceptive feedback loops in the limbs might, during fatigue, compensate sufficiently to maintain adequate accuracy in postural contractions, but that the fall in loop gain resulting from fatigue would cause deterioration in very highly skilled tasks, such as the fingering of notes on a violin. It is known, for example, that tremor increases during acute fatigue of a muscle (Sutton, personal communication). While, however, it seems that considerable muscular fatigue occurs with the violin, loss of intonation, which is a very sensitive index of inaccurate performance, was not noticed in experiments with the ischaemic arm until just before the fingers became too weak to press down the strings at all. Analogous results were obtained as regards timing, touch and expression on the piano.

In summary, these results suggest that the limit of muscular endurance may be nearer than is imagined even in light skilled tasks. Muscular fatigue is likely to be of importance in causing a sudden breakdown of performance when working near the limits of reserve. The present indications are that it will not be of significance at other times, owing, perhaps, to the excellence of the muscle's proprioceptive servo-control, which compensates automatically for fatigue. It is difficult to guess, without experiment, which tasks do strain the reserves of the muscles they use, for until the reserves are gone there is little or no subjective sense of fatigue. Nor do there appear to be relevant observations in the literature; in most work on fatigue of skilled movements (e.g. Bartlett, 1953) little attention has been given to the muscular factor.

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MUSCULAR EFFORT

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1 Sprint running
2 Middle-distance running
3 Long-distance running
4 The rise in ventilation
5 Oxygen transfer in the lungs
6 Increase in cardiac output
7 Other factors
References

Nervous, chemical and physical influences resulting from muscular contraction play a large part in the integration of muscular effort. The facet of this integration strained by muscular effort depends on the nature of the effort. The physical capacity of an individual is limited by the speed of contraction of muscle on the one hand, and by the force of contraction on the other. As a muscle approaches its maximum force the velocity of contraction falls; the maximum force, for example, in weight-lifting, is determined by the number and state of development of the active muscle fibres, these, in turn, being affected by the training to which the muscle has been subjected. At the opposite end of the scale, in sprinting and throwing missiles, lies the effort in which speed, rather than force, is the decisive factor.

Running is peculiarly suited to a study of the factors involved in muscular effort. Unlike many sports involving muscular effort, an absolute breaking point is reached, unaffected by those qualities of "touch" and "eye" which make objective measurement difficult and depend on neuro-muscular co-ordination. Record performances in athletics may not often be adequately controlled, but they represent innumerable tests of maximal muscular effort. Consideration of the limiting factors in running at different speeds throws valuable light on the physiology of muscular effort.

Hill (1925) was the first to plot speed against distance for world running records. Figure 1 shows the decline in speed as the distance run increased, plotted on the basis of world records in 1955. Though Hill made no attempt to derive any mathematical equations correlating the decline in speed with the limiting factors responsible, others, Lietzke (1954) and Henry (1954) for example, have attempted to deduce the relationship between the physiological mechanism and the rate of energy liberation. Henry regarded oxygen requirement as the sum of two exponential terms, oxygen debt and intake, and has derived equations of motion from acceleration and fatigue factors, which describe the speed and position of an athlete running at any time at a steady pace or "all out". But it is to be doubted that these empirical studies will throw much light on the physiology of muscular effort.

The quantitative assessment of the factors which are limiting might be possible by a series of experiments in which the subject would run to exhaustion at different treadmill speeds. This would be a formidable task—for the subject as well as the experimenter—and has not so far been undertaken. Hence it is not yet possible to define precisely the speed of running at which each limiting factor is maximally operative, but the nature of these factors when running at three different speeds will be discussed.

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1. Sprint Running

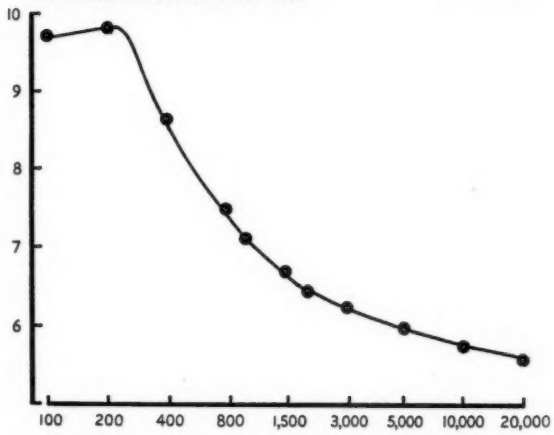
In the running of short distances, oxygen uptake is unimportant because there is insufficient time for transport of oxygen from the lungs to the muscles. A sprinter may even with advantage hold his breath in a 100-yard race, since a rigid thoracic wall provides the basis for the contraction of his limbs. Nor is the accumulation of chemical breakdown products limiting over very short distances, because here speed does not fall as the distance run increases. Respiratory and circulatory adjustments are necessary only for the recovery when products of the exercise are oxidized.

In sprinting, the body resembles a machine whose speed depends on the ratio of propelling force to resistance. Each foot draws and then drives the body forwards, the horizontal component of the thrust producing the runner's acceleration. The body is driven as it were, by pendulums worked by muscular power instead of gravity. By flexing the rear leg as it is drawn through, the length of the pendulum is reduced so that the movement is completed more quickly and with less effort. Powerful arm movements are necessary to counter-balance leg movements. Ground friction and wind resistance may be estimated and inertia measured by the time taken to reach maximum speed. The total energy is mainly utilized in acceleration and deceleration of the limbs, overcoming their viscosity. The rate of energy expenditure in running at maximum speed is about 13 horse-power for an average man. Fenn (1930) has calculated that the rate of effective mechanical work is about 3 horse-power, giving an efficiency of more than 20%.

2. Middle-Distance Running

Over the range of longer sprints and of middle-distance races there is a steady fall of speed with the distance run. The

FIG. 1. DECLINE IN SPEED WITH INCREASE IN DISTANCE RUN, PLOTTED ON THE BASIS OF WORLD RECORDS IN 1955



supply of energy is partly aerobic and partly anaerobic; the accumulation of the products of muscular contraction is a major factor limiting the performance. Hill, Long & Lupton (1924) suggested that the oxygen absorbed after the end of such exercise in excess of the resting oxygen uptake could be regarded as an " oxygen debt ". By comparison with isolated muscle, lactic acid was thought to be the " security " given for payment of the oxygen debt. There are several obstacles to accepting this theory in its simplest form. Margaria, Edwards & Dill (1933) have shown that no extra lactic acid appears in the blood during and after exercise involving oxygen debts of less than about 2.5 l. Also, there is no simple correlation between the oxygen uptake after exercise which falls rapidly, and the slow reduction in blood lactate; this latter is probably mostly taken up by heart and liver, but some by the muscles. The increased oxygen uptake after short maximal exercise is probably required to produce energy by oxidation of some compound or compounds (which may not be lactic acid) for re-synthesis of the energy-rich bonds resolved by contraction, which may have accumulated in exercise.

In this range of muscular effort, steady running may not be, as postulated by Hill, the most efficient. Runners seem to achieve their best times in the 440-yard race, for example, by running the first part of the race considerably faster than the second. Hill's " constant speed " hypothesis involves the assumption that the anaerobic and aerobic mechanisms of energy production are independent of one another. However, as lactate accumulates, the muscle pH falls and it is possible that this in itself affects the efficiency of the aerobic mechanism.

3. Long-Distance Running

As the distance run increases, anaerobic mechanisms, although contributing a proportion of the total energy required especially at the onset of exercise, become progressively less important. For example, anaerobic mechanisms probably contribute nearly half the total energy required in running a mile but less than a quarter of the energy required in running three miles. For longer distances the work demanded of the muscle is within the range of speed and force which it can easily sustain. Such effort is limited mainly by the rate of supply of metabolic necessities to the muscle, and by the removal of the products of muscular contraction. Long-distance running is of great physiological interest because it provokes the greatest respiratory and circulatory adaption of which the body is capable.

Analysis of the limits of performance in long-distance running requires an extension of our knowledge of " steady state " exercise. For equally efficient runners, the speed of movement depends on the maximal oxygen uptake which can be maintained. The blood lactate level falls during steady exercise (Bang, 1936), though this was disputed by Eskildsen (1947), and small steady muscle pyruvate and blood lactate levels in excess of the resting values probably represent merely a new balance between rates of formation and removal.

During such exercise, the oxygen consumption of muscles (calculated from oxygen uptake in relation to body muscle weight) may increase some fifty times. Our knowledge of the effective limits of oxygen transfer from air to muscles is incomplete, but new light has been shed on some aspects of this problem. Recent experiments investigating the rise in ventilation, the oxygen transfer across the pulmonary epithelium, and the increase in cardiac output, will serve to illustrate these trends.

4. The Rise in Ventilation

The rise in ventilation during exercise was explained by Haldane & Priestley (1905) in terms of an increase in blood partial pressure of CO_2 (pCO_2) and hydrogen-ion concentration, but this view was challenged by Krogh & Lindhard (1917) on the grounds that the Haldane-Priestley method of alveolar sampling did not reflect the arterial CO_2 during exercise. It seems probable that a modification of the Haldane-Priestley method gives alveolar values for gas tensions which approximate closely to those found in the arterial blood and that a rise of alveolar pCO_2 often takes place during exercise. However, most arterial pCO_2 measurements during exercise show little if any change. In recent experiments (Bannister, Cunningham & Douglas, 1954), the rise of alveolar CO_2 in two athletic subjects was much greater than that in two non-athletic subjects in whom emotional factors aroused by unaccustomed exercise may have contributed relatively more to the rise in ventilation. We have suggested an explanation for the discrepancy between the observed rise and that which would be expected if CO_2 were the sole factor producing the hyperpnoea of exercise.

Cotes (1955) has shown that, during oxygen breathing, ventilation is controlled by the effects of CO_2 tension and the rise of body-temperature and that moderate exercise influences ventilation only indirectly by its effect on these two variables. Nervous stimuli from the working limbs (Harrison, Calhoun & Harrison, 1932; Grodins, 1950) are probably of importance only in producing rapid adjustments to the changing conditions which occur at the start of exercise.

As the speed of running increases, another stimulus to respiration becomes active because suddenly enriching the inspired air with oxygen produces a fall in ventilation with a very short latency. Asmussen & Nielsen (1946) postulated that a substance produced by ischaemic muscles stimulates the chemo-receptors and is rapidly destroyed in the presence of oxygen. An alternative explanation in terms of known factors is that when oxygen is added to the inspired air, the chemo-receptor drive caused by arterial anoxaemia is removed (Bannister & Cunningham, 1954). The only direct measurements of arterial oxygen tension during heavy work are those of Lilienthal, Riley, Proemmel & Franke (1946). They found that it fell from 94 mm. Hg at rest to 73 mm. Hg during three experiments on Riley who exercised at moderately severe intensity.

Recent experiments (Cormack, Cunningham & Gee, 1955) provide valuable indirect evidence supporting this hypothesis from re-breathing experiments at rest. These showed that, in the presence of a large excess of CO_2, very powerful stimulation from oxygen lack may occur when the alveolar pCO_2 is well above 100 mm. and that the influence of these stimuli may not be simply additive as Gray (1950) has suggested. Cunningham & O'Riordan (1956) have demonstrated the influence of temperature on the CO_2 stimulus at rest. The results of these experiments suggest that ventilations at rest similar to those in exercise may be produced by the interaction of several stimuli all probably operative in exercise, though the qualitative nature of the interaction is as yet incompletely understood.

Thus there is growing evidence that arterial anoxaemia occurs in severe exercise and causes a further rise in ventilation when other respiratory stimuli are maximally active. The maximum ventilation, which varies with the individual but sometimes exceeds 100 l./min. at standard temperature and pressure, is probably not limited by mechanical factors, as it is considerably less than the maximum ventilation during forced breathing at rest.

5. Oxygen Transfer in the Lungs

For maximal oxygen uptake in the lungs adequate ventilation during exercise must be balanced by adequate circulation of blood through the lungs. The effect of uneven distribution of blood and air to different parts of the lungs, suggested by Haldane & Priestley (1935), has been defined theoretically and measured by Riley & Cournand (1951), who expressed it in terms of a complete " venous shunt " which at rest probably amounts to 3% of the total blood flow. These advances have been made possible by new methods of blood-gas analysis and by the technique of cardiac catheterization allowing direct measurement of the gas content of blood entering the lungs. Local over-ventilation and under-perfusion (and vice versa) increase the difference between the oxygen tensions of the alveolar air and of the blood leaving the lungs. The smallness of the alveolar arterial gradient at rest indicates the delicacy of the adjustment of alveolar ventilation to blood flow. It seems reasonable to assume that during maximal exercise this adjustment is to some extent sacrificed in favour of greater blood flow. During exercise there may be cessation of pulmonary vasoconstriction or at any rate loss of active distribution of blood, so that arterial anoxaemia may exist when atmospheric air is breathed. Anoxaemia may exist even when the level of oxygen in the inspired air is raised, since it has been shown that increasing the level of oxygen in the inspired air above 33% causes further reduction in ventilation during exercise (Bannister & Cunningham, 1954).

The diffusion capacity of the lungs for oxygen during exercise (the ratio of the gas transferred to the gradient of oxygen tension between the alveoli and the arterial blood) has been measured in two ways. Riley and Cournand have used a direct method by lowering the inspired oxygen tension to the region of the blood dissociation curve, where changes of tension can be more easily measured. Bates, Boucot & Dormer (1955), adapting the CO method of Krogh & Krogh (1910), have recorded a similar increase in exercise. Riley and also Cournand and Bates explain the increase in diffusion capacity by an increase in the pulmonary capillary bed in exercise. Bates has suggested that, in athletes with a high diffusion capacity, changes in permeability of the pulmonary epithelium and capillary bed may play a part. This concept may require modification in the light of Roughton's recent work (Gibson, Kreuzer, Meda & Roughton, 1955) on the kinetics of the reaction of CO with haemoglobin. He suggests that the formation of carboxyhaemoglobin may be slow enough to retard the uptake of CO from the alveolar air. If this is so, more work will be required to assess the contribution of membrane resistance and the slowness of the carboxyhaemoglobin reaction to the over-all observed limitation of diffusion as measured in vivo.

Recently the work of Lee & Du Bois (1955) has suggested another possible cause of reduction in arterial O_2 tension during exercise. By enclosing a subject's whole body in a plethysmograph and measuring the uptake of nitrous oxide by the lungs, they have succeeded in measuring changes in pulmonary capillary flow. They have shown that pulmonary capillary flow is pulsatile—the peak flow being five times the minimum flow. Transfer of N_2O, like that of CO_2, probably takes place at the beginning of the capillary. Lee and Du Bois' studies of oxygen uptake in the lungs are incomplete, but it is possible that when blood flow is maximal, as in exercise, oxygen, which diffuses more slowly, is incompletely taken up during the peak flow, and a jet of blood with lowered oxygen tension passes from the lung capillary at each systole.

6. Increase in Cardiac Output

If maximal oxygen transfer in the lungs is maintained, the rate of supply of oxygen to the muscle depends on the rate of circulation of the blood. Knowledge of circulatory control at rest is based more than that of respiratory control on animal experiments and is therefore less advanced. There is now general agreement that the cardiac output, measured by the indirect Fick, foreign gas or dye methods may rise to more than 30 l./min. during severe exercise (Asmussen & Nielsen, 1955). In trained subjects the arteriovenous oxygen difference remains low and constant at increasing rates of work, and a large blood flow is maintained at low pulse rates because of the larger stroke volume (Christensen, 1931). The vascular resistance in the muscles is presumably lower in athletes than in normal subjects because, in spite of the higher output in athletes, the arterial pressure is not raised (Bock, Vancaulaert, Dill, Fölling & Hurxthal, 1928).

In studying the circulatory changes during exercise, it has been difficult to compare the results of experiments on animals and man. Starling's theory of the increase in cardiac output in exercise, based on the heart-lung preparation (Patterson & Starling, 1914), requires an increase in the diastolic volume of the heart. McMichael & Sharpey-Schafer (1944) have shown that there is a linear relationship between stroke volume and atrial filling pressure when the latter is varied passively. It seems, however, that in human subjects during exercise the stroke volume may rise without an increase in the diastolic volume (McCrea, Eyster & Meek, 1927) or the central venous pressure (Landis, Brown, Fanteux & Wise, 1946). Studies on ventricular function may provide the explanation for this apparent negation of Starling's principle. McMichael (1952) first suggested a series of different curves relating cardiac work to diastolic filling, for normal, failing and hypertrophied hearts in human subjects. Sarnoff (1955) has, for anaesthetized dogs with complete circulations, plotted ventricular work against mean atrial pressure (the indices which Starling's principle states to be directly related) on each side of the heart. Changes in the state of the myocardium produce different members of a family of curves, but Sarnoff has shown that a consistent relationship exists between atrial pressures and ventricular stroke work on the same, but not on the opposite, side of the heart. Future experiments may throw light on the theory of Asmussen & Nielsen (1955) that the increased stroke volume during muscular work is produced by more complete emptying of the ventricles during systole called forth by nervous and possibly hormonal factors.

The reflex control of circulation during exercise is inadequately understood. Though impulses have been recorded from receptors that probably correspond to those formerly thought to initiate the Bainbridge reflex, the central effect of

such afferents remains unknown. It is possible that there are reflexes arising in the right heart and pulmonary vessels which are sensitive to anoxia (Mills, 1944). Future work may elucidate the effect on respiratory and circulatory control of receptors in the heart and lungs, which have been shown to cause profound changes in blood pressure, heart rate and breathing (Dawes & Comroe, 1954).

7. Other Factors

It is probable that the part played by the autonomic system in the smooth integration of exercise has been under-estimated. The changes of blood flow to skin, splanchnic areas and active muscles during exercise are well known. During exercise, however, the autonomic system also aids the regulation of body-temperature. Heat liberated in contracting muscles—which increases the efficiency of these contractions—is at first retained, yet when overheating occurs, heat-loss mechanisms are augmented. It is noticeable that, during running on a treadmill, the exercise becomes easier to maintain when the initial cutaneous vasoconstriction passes off and sweating occurs. Both adrenaline and noradrenaline are known respiratory stimulants (Whelan & Young, 1953). It is possible that the sympathetico-adrenal system may exert some regulating effect on the respiratory and circulatory adaptation during exercise.

In the future, it may be discovered that the sympathetico-adrenal system plays a part in extending momentarily the known limits of physiological adaptation. The effect of adrenaline injections in prolonging muscular exercise in fatigued dogs has long been known (Campos, Cannon, Lundin & Walter, 1928). More recently von Euler (1952) showed that, in a well-trained human subject, noradrenaline excretion was slightly increased in moderate exercise. However, when the oxygen consumption rose above 2.4 l./min. the noradrenaline excretion rose to approximately twenty-five times the resting level. The stimulus to noradrenaline excretion in exercise, whether nervous or chemical, is unknown, but it is likely that a significant fall in arterial pO_2 occurs at this intensity of running.

* * *

In conclusion, it seems that the muscular effort of running is limited by two factors. The first is the maximal contractile activity of muscle, which limits speed in sprinting. The second is the rate of supply of oxygen and metabolites to muscles and the removal of the products of muscular contraction. In "steady state" running, efficient oxygen transport to muscles is achieved by a physiological integration causing increases of pulmonary oxygen intake and transfer, cardiac output and muscle blood flow. Yet in "all-out" running in which adequate carbohydrate is available, oxygen supply fails to keep pace with requirements and there is evidence that the arterial pO_2 falls. Oxygen supply is the weak link. But it seems academic to argue whether this failure occurs because the alveolar-arterial gradient for oxygen is increased or because blood is not circulating fast enough. The muscular effort in long-distance running appears to be limited by cardio-respiratory failure as a whole and not by premature failure of any single part of the integration.

Yet the difference between athletes lies not entirely in differences of cardiac output or diffusion capacity; it lies rather, I suspect, in their capacity for mental excitement, which brings with it an ability to overcome or ignore the discomfort—even pain—in muscles and the brain which is probably caused by ischaemia and the consequent changes of blood lactate concentration and pH. Though physiology may indicate respiratory and circulatory limits to muscular effort, psychological and other factors beyond the ken of physiology set the razor's edge of defeat or victory and determine how closely an athlete approaches the absolute limits of performance.

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FAMILIAL PERIODIC PARALYSIS

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1 Clinical features
2 Relation of potassium to the paralysis
3 Theories as to causation
  a Metabolic abnormality
  b Membrane abnormality
4 Electromyographic findings
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A feature of advancing medical knowledge has been the recognition that many apparently well-defined diseases are, in fact, syndromes caused by differing pathological or biochemical processes. Familial periodic paralysis is no exception. Talbott (1941) in his review of periodic paralysis recognized that the paralysis was an exaggerated physiological response, analogous to cramps or tetany, which could be brought about in a variety of ways, although at the time he naturally considered a lowered serum potassium (K) to be a constant accompaniment of the paralysis. Later work has shown that some of the cases of sporadic periodic paralysis are due to a gross increase in the serum K (Finch & Marchand, 1943; Bull, Carter & Lowe, 1953), and in the last few years it has become increasingly plain that, even in the hereditary condition, some of the families develop a clinically similar type of paralysis associated with a normal serum K and without response to K therapy (Tyler, Stephens, Gunn & Perkoff, 1951). A patient from such a family (McArdle, unpublished) showed, during attacks, a slightly raised serum K which fell on recovery. He tended also to have an increased excretion of K during the paralysis, in contrast to the low levels of K excretion found in the typical case. A similar family has been described by Sagild (1954). This striking difference in regard to K metabolism, coupled with a lack of response to K therapy, clearly separates these families from those with the classical type of periodic paralysis, and would seem to entitle them to a separate name of their own. This review will be concerned only with the type associated with a fall in serum K, and with a response to K therapy.

1. Clinical Features

It used to be all too common for the attacks of paralysis to be diagnosed as hysteria. Those days may have passed, but the disease is sufficiently rare to merit attention being drawn to some of the more important clinical features. The condition is characterized by attacks of flaccid paralysis affecting the limbs and trunk muscles, and sparing the facial, respiratory and pharyngeal muscles in all but the most severe attacks. When paralysis is complete, the tendon reflexes are absent and there is loss of muscle excitability to direct stimulation. Consciousness and sensation remain unimpaired. The attacks vary in severity and duration, but rarely last for more than two days, the average duration being about six to eight hours. Males are more frequently affected than females. The first episode commonly occurs in the second decade but, with increasing age, the number of attacks tends to diminish and often to cease. If recognized sufficiently early, the paralysis may be relieved or postponed by taking exercise and "walking it off", but it is very prone to commence in the early hours of the morning, and too often the patient awakes to find himself unable to rise from his bed. Exposure to cold may provoke attacks in some patients, and an arm left lying outside the bed-clothes may be more severely paralysed than the other limbs.

A number of the families afflicted with the condition (as for example, those described by Oppenheim, 1891; MacLachlan, 1932; Bickerstaff, 1953) are liable also to be affected by a permanent and progressive form of weakness and wasting of certain muscle groups with loss of the reflexes. Biemond & Daniels (1934) regarded the condition as a "spinal muscular atrophy", but it is more probably myopathic in origin.

2. Relation of Potassium to the Paralysis

The first clear insight into the cause of the paralysis was given by Aitken, Allott, Castleden & Walker (1937) who showed that the serum K fell to low levels during an attack, and that the paralysis could be cured by giving adequate amounts (5–10 g. or more) of KCl by mouth. Biemond & Daniels (1934) had earlier found the serum K low in an attack, but did not grasp the significance of this. The findings were confirmed by a number of workers, including Allott & McArdle (1938) who showed that K was retained in the body during the attacks. Since the fall in serum K was not due to renal loss, it was presumed that K had entered the cells. They also showed that the threshold level for weakness varied between patients. By repeated injections of adrenaline, the serum K of a normal subject was lowered without any weakness to levels which, in patients with this disorder, would have been associated with severe paralysis. They concluded that the low serum K was only one factor in producing an attack, an abnormality of the neuromuscular apparatus being another essential part. Later work has reinforced this view. Hypokalaemic paralysis due to potassium deficiency or occurring in association with hyperthyroidism (Weissman, 1952; Lindner, 1955) is now a well-recognized clinical entity, but the level of serum K at which weakness supervenes is usually lower than the threshold level of about 3–3.5 m-equiv./l. in the familial condition. In two of three cases of familial periodic paralysis personally studied, the weakness occurred at 3.5 m-equiv./l. or even higher, whereas, in their studies on experimental K deficiencies, both Black & Milne (1952) and Fourman (1954) reduced their serum K levels below 3.2 m-equiv./l. without experiencing any gross weakness.

It has been repeatedly shown that during attacks there is a marked positive balance of K (Allott & McArdle, 1938; Gammon, Austin, Blithe & Reid, 1939), the urinary K excretion falling to very low levels as the concentration in the plasma falls. The K presumably moves into the cells (Danowski, Elkinton, Burrows & Winkler, 1948), but the mechanism responsible is not fully understood. A previous depletion by losses from renal or gastrointestinal tracts is an obvious theoretical possibility, but it seems highly unlikely that this could be the sole or even an important factor in the genesis of the attacks. All the evidence derived from the

experimental production of K deficiency in otherwise normal men (Black & Milne, 1952; Fourman, 1954) and from instances in which paralysis has been observed in hypokalaemic states (Milne, Stanbury & Thompson, 1952; Fourman & McCance, 1955; Fitzgerald & Fourman, 1955) indicates that a severe depletion is necessary before paralysis occurs. There is certainly no evidence that such a severe depletion is encountered in familial periodic paralysis. Thus a patient studied by McArdle and Merton was in K balance, and his total- and cell-exchangeable K, determined with ^{42}K, was normal (McArdle & Merton, 1952). However, this was on a high K diet. When he was placed (unpublished studies) on a low normal K diet he lost about 7 m-equiv. of K per day, though, as he was on this diet for only ten days, it is possible that he might later have reached a new equilibrium. On two occasions, whilst he was on a normal K intake, 25 mg. of adrenocorticotrophic hormone (ACTH) injected intramuscularly precipitated severe or very severe attacks, whereas the same or greater amounts given on the higher intake had slight or no effects. The increased susceptibility on the normal intake, however, could not have been due to any generalized K depletion, since it was apparent within a few days of starting the lower K diet, at a time when the body K could have been decreased by only about 1%. It would appear, therefore, that in this patient a mild sudden depletion of K brought about by diet, deoxycorticosterone acetate (DOCA) or ACTH was enough to trigger an attack, but the nature of this abnormality is very poorly understood; it must be a disorder of K transport, in or out of the cell, and can therefore be considered to be the result either of a metabolic defect within the cell, drawing K into the cell, or of an altered permeability of the cell membrane to K. Such a defective membrane must be caused by a local or more general metabolic abnormality, but, in discussing how the paralysis is caused, it is convenient to consider a defect in the muscle membrane under a separate heading.

3. Theories as to Causation

a. Metabolic Abnormality

If a metabolic error is in fact responsible for the abnormal shift of K into the cells, it seems most likely that it is concerned with carbohydrate metabolism. Thus paralysis commonly follows a large carbohydrate meal, and it has almost become a routine practice in investigating the condition to attempt—usually successfully—to induce paralysis either with glucose, or better with insulin and glucose. It is significant, also, that in some patients severe and long-lasting attacks can be induced with relatively small doses of adrenaline. Further, paralysis frequently occurs during the night following unusually heavy exertion. These facts raise the possibility that a disturbance of carbohydrate metabolism is responsible for the unusual shifts of K into the cells, especially since it is known that the deposition of glycogen is accompanied by the movement of K into the cell (Fenn, 1939). However, the work of McQuarrie & Ziegler (1952) has made the association rather less definite. They studied the relation between the types of diet and the susceptibility to paralysis in a boy normally subject to very frequent attacks. They confirmed the deleterious effect of a high-carbohydrate diet (total calorie intake 2,263–2,861), and showed that, as the proportion of carbohydrate in the diet was increased, it was necessary to give large and increasing amounts of K salts in order to prevent attacks. They also concluded that a high-protein diet (total calories 1,465–1,645) had a beneficial effect in preventing attacks and that a high-fat diet (2,666–3,334 calories) was deleterious. However, their findings on the diets high in protein and fat could be equally well, if not better, explained as the result of a deficient or excessive calorie intake respectively, since this patient was virtually free from attacks so long as the diet, irrespective of composition, contained less than 2,000 calories, and he had none during a period of starvation.

While it is clear that measures involving carbohydrate metabolism can provoke attacks, it has not been proved that the metabolism of carbohydrate is itself abnormal in these patients. To obtain information on this point McArdle and Merton followed changes in blood pyruvate and lactate and in the serum inorganic phosphate (PO_4) during and between attacks. During a severe spontaneous nocturnal attack the blood pyruvate was raised, and it fell with recovery, a finding the reverse of the usual diurnal rhythm of serum PO_4 (Stanbury, personal communication). A good correlation was found between the levels of the serum K and inorganic PO_4, the two tending to rise and fall together both during and between attacks. Blood pyruvate levels were inversely correlated with the serum K. These correlations were not found in a normal subject, but the findings can scarcely be regarded as being more than indications of an abnormality in carbohydrate metabolism.

Further evidence of a metabolic defect was obtained, between attacks, by following the changes in K, PO_4, pyruvate and lactate in blood coming from the forearm muscles on releasing the occluding cuff at the end of a period of ischaemic work. By measuring the blood flow after the same amount of work, and by combining these values with the blood or plasma changes, it is possible to draw up a rough balance sheet of the muscle exchanges following ischaemic work. These changes were then related to the amount of work done. The muscle commenced to take up K very soon after the release of the occluding cuff, and continued to do so throughout the 20 minutes during which the exchanges were followed. The fall in serum K of the blood coming from the muscle, and the actual amount taken up, were considerably greater than any of the control values, the amount taken up per kg.m. of work done being about fifteen times greater than the mean control value. Both the muscle output of phosphate during the first minute and the succeeding uptake were about five times greater than the mean values for the corresponding exchanges in normal subjects; pyruvate and lactate outputs were two-and-a-half times the mean normal output. Since this abnormal uptake of K, and abnormal exchange of other substances concerned with intermediary carbohydrate metabolism, can be demonstrated between attacks, it seems reasonable to presume therefore that during attacks the muscles are also directly concerned in the fall of serum K.

These findings—coupled with the abnormal K responses to glucose, to insulin and glucose, and to adrenaline—could be tentatively interpreted as indicating a partial block at the hexose phosphate stage of carbohydrate metabolism (Allott & McArdle, 1938). Such a biochemical lesion would result in the excessive accumulation of hexose phosphates both during the breakdown and during the synthesis of glycogen, since the accumulation of these indiffusible anions causes an inward flux of K (Boyle & Conway, 1941). Such a block might be sufficient of itself to cause the abnormal shifts of K, but it could have additional effects mediated through the " sodium pump ". The active extrusion of sodium (Na) from

the cell by the pump is thought to be linked with the active transport of K into the cell (Hodgkin & Keynes, 1955a). It is dependent on metabolism and is slowed if this is inhibited. However, the block envisaged is partial, and marked inefficiency might occur only during heavy exercise. Under these circumstances Na would be retained, and K would leak from the cell. Restoration of efficiency with rest would lead to reversal of these ionic movements and a greater influx of K than would otherwise have been the case.

b. Membrane Abnormality

Neel (1928) and Zabriskie & Frantz (1932) showed that either local or general attacks of weakness could be induced by exposure to severe cold, and, in the patient studied by Bickerstaff (1953), gross local weakness followed immersion of the hand in water at 14° C. at a time when the serum K was normal. McArdle and Merton showed that the gross weakness induced in their patient by immersing the arm in water at 20° C. for twenty minutes could not be due to a local lowering of the serum K, since the serum level in the blood coming from the cooled muscles was 4.6 m-equiv./l., a value that was 0.2 m-equiv./l. higher than that coming from the opposite arm kept in a bath at 37° C. These observations are important, since they show not only that the muscles themselves are at fault, but that under suitable conditions gross weakness can be induced in the absence of any change in the serum K. The findings clearly reinforce the view that the fall in serum K is only one factor, though a very important factor, in the paralysis, and that some other defect in conduction of the impulse along the muscle fibre must be present. A disorder of the muscle membrane is the most probable cause of such a failure in conduction, and, since the evidence for a block in carbohydrate metabolism is incomplete, the alternative possibility of a primary disorder of membrane function must be considered.

A disturbance of membrane permeability could conceivably result in periodic paralysis by interfering with either the active or passive transport of K. It is impossible in the present state of knowledge to make any worth-while guess as to which might be affected, but, in order to draw attention to the importance of investigating this matter, one example will be given of a possible mechanism by which an exaggerated fall in serum K might occur.

In the passive transfer of K across the membrane (see Hodgkin & Keynes, 1955b), the direction of the net movement of K depends on the difference between the equilibrium potential for K (E_K) and the actual potential difference across the membrane (E). E_K depends on the ratio of the internal to the external concentration of K, i.e. $\frac{[K]_i}{[K]_o}$. With moderate or high $[K]_o$ it appears that the resting membrane potential is essentially a K potential with E equal to E_K. This is because the permeability to K, under these conditions, is greater than to other ions, so that the other ions do not make an appreciable contribution to the membrane current. When $[K]_o$ is reduced, however, the rising membrane potential causes a reduction in K permeability (Hodgkin & Keynes, 1955b), so that the ratios of other ions across the membrane, notably chloride (Cl), begin to play a significant part in determining the height of E. If the Cl ratio at this time is less than the K ratio, E will fall below E_K, resulting in a net outward movement of K. This was the situation in the isolated sepia axons investigated by Hodgkin and Keynes. If, however, when Cl permeability becomes significant, the Cl ratio is higher than that for K, the membrane potential will rise above E_K, and the effect of lowering the external K at that time will be to cause K to be drawn into the muscle. In this way, as suggested by Merton (personal communication), a normal demand for K in some other part of the body might result in excessive falls in serum K—particularly in the prolonged falls observed in these patients, since the fall in K would tend to perpetuate itself as a regenerative cycle.

The proposed alterations in Cl are equivalent to the well-known chloride shift in red corpuscles. They might also, therefore, be affected by changes in acid-base balance or by abnormal permeability changes induced by certain hormones, or by exercise in this condition. An increase in Cl ratio due to a shift of Cl from the cell is, indeed, part of the mechanism by which it is supposed that the increase in indiffusible anions caused by a carbohydrate block induces the uptake of K (cf. Boyle & Conway, 1941). This theory, in part, presupposes the presence of Cl inside the cell. There is evidence for this in nervous tissues (Keynes & Lewis, 1951; Coombs, Eccles & Fatt, 1955), but the opposite view is still held and it is difficult to prove one way or the other. Whether the normal human muscle cell does, or does not, contain Cl, it is not hard to conceive that a defective membrane might become permeable —if only temporarily—to this ion. That a disorder of the membrane is likely would seem to follow not only from some of the clinical features, but also to explain some of the electromyographic and other findings.

4. Electromyographic Findings

A characteristic feature of periodic paralysis, first noted by Hartwig in 1875, is the complete failure of the muscles to respond to direct stimulation during a severe attack. This showed that the muscles are primarily involved, a conclusion supported by all subsequent workers with the exception of Pudenz, McIntosh & McEachern (1938). In recent years, the nature of the paralysis in muscle has been studied by stimulating the motor nerves electrically and recording the muscle action potentials. During paralytic attacks the voltage of the muscle action potentials is reduced, showing that conduction is blocked at some point, perhaps at the neuromuscular junction (Johns, Liljestrand, Grob & Harvey, 1955). There is also a change in shape of the action potentials, which have been described as " monophasic ". This must be attributed to some peculiarity of conduction along the muscle fibres (Eichler, Jantz & Jung, 1940; Gammon, Harvey & Masland, 1941; Merton, unpublished). Merton points out that during attacks the lowered serum K will increase the ratio $\frac{[K]_i}{[K]_o}$ and lead to a raised membrane potential. Such a hyperpolarization would raise the electrical threshold of the muscle fibres and may account for the conduction block. The actual membrane potential will depend not only on the level of serum K, but also on other circumstances such as the Cl ratio (as discussed above). This might account for the fact that patients with this disease often become paralysed at a level of serum K which is without effect on a normal subject. Merton has also observed that the pathological action potential is less effective at activating the contractile mechanism; thus, relative to the height of the action potential, the tension developed during a single mechanical twitch of the muscle is

much smaller than in healthy subjects. This is probably to be interpreted as a result of the abnormality in action potential form (see paper by Merton, p. 219 of this number of the Bulletin).

From the alternative views put forward, it must be evident that our knowledge of the fundamental disorder in the familial type of periodic paralysis is still very deficient ; but there are two lines of approach which offer hope of a better understanding of the disorder. The first is the investigation of those enzyme systems, such as phosphoglucomutase, concerned in the metabolism of carbohydrate at the hexose phosphate level; the second is the determination of the resting membrane potential, both during and between attacks.

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CHANGES IN MUSCLE AFTER DEATH

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1 Physical changes during rigor mortis
2 Chemical changes during rigor mortis
3 Relation of the physical to the chemical changes
4 The temperature coefficient of the chemical and physical changes
5 Other physical and chemical changes associated with stiffening
References

Of the diverse changes which occur after death the most obvious is the stiffening of the corpse, recorded from the earliest times and recognized as arising specifically from stiffening of the muscular tissues. It is this change, rigor mortis, and the underlying chemical changes, with which we shall be mainly concerned here.

The process of stiffening can be estimated in terms of the extension of the muscle by an applied load (Bate-Smith, 1939; Bate-Smith & Bendall, 1947, 1949), from which either the extensibility or the modulus of elasticity can be calculated. It is found that the hardness, which can be regarded as proportional to the modulus of elasticity, increases ten to forty times during the course of rigor mortis.

Although the physical changes can be expressed simply in this way, the chemical changes—which are more difficult to describe and estimate—were long misunderstood and have only recently been elucidated. Thus, for many years it was thought that the stiffening was due to coagulation of the muscle plasma either spontaneously by a process akin to the coagulation of the blood, or by precipitation of the proteins by the lactic acid produced after death. The former theory seems to have originated with Kühne in 1864, the latter with Schipiloff in 1882, and these views have persisted in one form or another until quite recently. That lactic acid was not necessarily involved had, however, been shown by the experiments of Claude Bernard as early as 1877, and of Hoet & Marks in 1926, on so-called " alkaline rigor " during which no acid was produced at all, yet the stiffening occurred as rapidly and as completely as in normal rigor. This led Hoet & Marks (1926) to postulate a third change, to which both stiffening and acid production could be related, and which has been variously supposed to be a loss of glycogen or of creatine phosphate from the muscle.

The true nature of this " third change " was not brought to light, however, until the importance of adenosinetriphosphate (ATP) in influencing the physical state of muscle had been made clear first by the classical discovery by Engelhardt & Ljubimova in 1939 of the ATPase activity of the structural protein myosin, and then by the discovery of actomyosin and its contractility in the presence of ATP by Straub and Szent-Györgyi (reported by Szent-Györgyi, 1945). These led directly to the demonstration by Erdös in 1943 that it was indeed loss of ATP from the muscle after death which was most closely related to the onset of stiffening.

The findings of Erdös were confirmed by Bate-Smith & Bendall (1947) who, in extending the original observation to muscles under a wider variety of physiological conditions, showed that the onset of stiffening was universally correlated with a loss of ATP. Similar results have been reported with whale muscle (Marsh, 1952a), horse muscle (Lawrie, 1953) and beef muscle (Marsh, 1954). Because of this wide correlation, it is clear that in describing further the conditions which determine the onset of rigor mortis we need consider only those chemical changes that control the level of ATP within the muscle.

1. Physical Changes during Rigor Mortis

Mangold (1927) followed the onset of stiffening by measuring the increase in hardness of the muscles with a sclerometer. Although this method is more akin to the qualitative test of stiffness by feeling the muscle with the fingers, it has the disadvantage that it is difficult to interpret the changes in terms of fundamental molecular structure. On the other hand, the changes of extension of a muscle with parallel fibres are easy to interpret and can be arranged to give a continuous record. Thus, Bate-Smith & Bendall (1948, 1949) have described an apparatus which alternately applies a load to the muscle for 8 minutes and removes it during the following 8 minutes, when the cycle is repeated until stiffening is complete. Typical records are given in fig. 1.

The method is based on the interpretation of the stretch–time curves of muscle given by Bate-Smith in 1939, who showed that three arbitrary phases could be distinguished, each of which had its characteristic exponential equation. Thus:

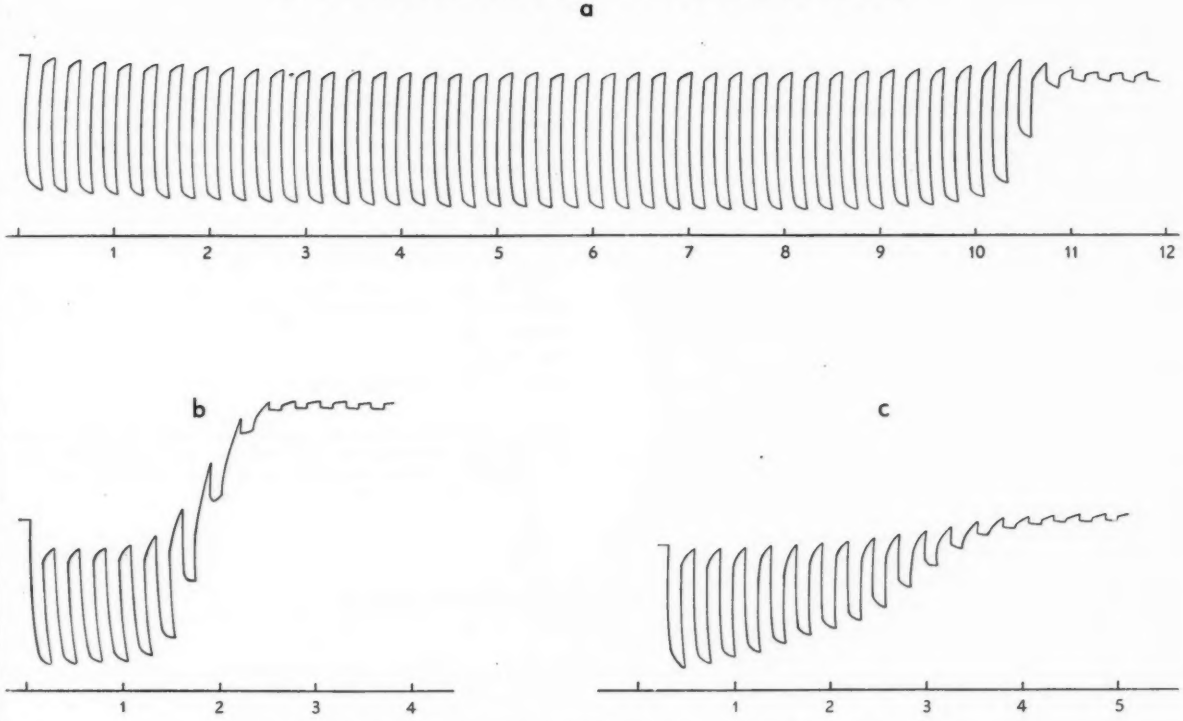
$$\text{Stretch in time } t = \underset{\text{seconds}}{A(1-e^{-k_1 t})} + \underset{\text{minutes}}{B(1-e^{-k_2 t})} + \underset{\text{hours}}{C(1-e^{-k_3 t})}$$

The slow component, C, can be almost eliminated by taking the stretch in the first 8 minutes only, by which time B is virtually complete. Bate-Smith calculated the modulus of elasticity or the extensibility from components $A + B$. This, however, has the disadvantage that it gives erroneous values for the extension when shortening occurs concomitantly with stiffening, as in record b (fig. 1). To avoid this, Bate-Smith & Bendall (1949) estimated the stretch in the first 15 seconds by extrapolation of the rapid portion of the curves; this effectively eliminates the distorting effect of the shortening.

Each of the records in fig. 1, although of differing duration, shows three main phases: a phase when the extension remains high and constant—the delay period; a phase of rapid diminution of extension—the rapid phase; and a phase where the muscle is in full rigor, and the extension has decreased ten-fold to forty-fold and thereafter remains unchanged for many hours. The factors which affect these phases will be discussed later.

Two further points should be noted here: the shortening during rigor, and the so-called " resolution of rigor ". As we have seen, considerable shortening sometimes occurs during the rapid phase of rigor (record b, fig. 1), but it should be made clear that this shortening is not a necessary concomitant of stiffening and rarely occurs under the normal conditions

FIG. 1. EXTENSION CHANGES IN RABBIT PSOAS MUSCLES



a: well-fed, immobilized rabbit; pH at beginning of record: 7.10; at end: 5.79
b: insulin-treated rabbit; pH 7.25 throughout
c: well-fed rabbit, not immobilized; pH at beginning of record: 6.52; at end: 5.92
Downward strokes: loading curves; upward strokes: unloading curves
Horizontal axis: time after death (hr.)
Time of one cycle of loading and unloading: 16 min.
Load: 50 g./cm.^{2} cross-section
Temperature: 17° C.
Initial extension: 5–8% of initial length
(Records were obtained with apparatus described by Bate-Smith & Bendall, 1948.)

of rigor at room temperature (10°–25° C.). It is dependent on temperature and pH, and is most marked at high temperatures and in exhausted animals (Bendall, 1951; Marsh, 1954). Its importance was stressed by Bendall (1951) because of its coincidence in time with the disappearance of ATP, but it is now of little further theoretical significance in view of the much clearer demonstration in model systems of the relation between shortening (or work done) and ATP split (cf. Weber, 1956). Moreover, shortening in rigor rarely develops more than $\frac{1}{2,000}$ of the maximum power of the fibre models (Bendall, unpublished). In passing, it should be made clear that this shortening has nothing to do with the so-called " excitability " of the muscle to an applied stimulus. Although the latter is known to pass off during the course of rigor, no study has been made of it in relation to the chemical and physical events to be described here.

The so-called " resolution " of rigor is of special importance in forensic medicine and for meat technology. Its mechanism, however, is obscure, because there is no evidence that any relaxation of stiffness ever occurs in isolated muscles, except as a result of energetic mechanical handling or bacterial decomposition. For example, a rabbit psoas, in full rigor, was held at 3.5° C. and then, for 5 days continuously, was lightly loaded and unloaded, alternately, at 8-minute intervals. At the end of this time, the extension had only increased from 100 on the first day to 130, whereas if full resolution had occurred it should have increased to about 2,000 (Bendall, unpublished). Marsh (1954) reports similar results with beef muscle held at 7° C. for 7 days. Thus, the resolution of rigor can have little to do with reversal of the extensibility change in the substance of the muscle itself, at least in the normal range of temperature between 10° and 25° C. In the human corpse, the effect is probably a combination of mechanical flexion of the joints and putrefactive softening of the tissues. It should not be confused with the " flabbiness " which often succeeds stiffening in isolated muscle, particularly at pH values below 5.70, but which is not accompanied by any increase in extensibility and which is probably due to

disruption of the sarcolemma of the muscle fibres. It is possibly this latter change, together with partial destruction of connective tissue, which is responsible for making meat tender during " hanging ".

2. Chemical Changes during Rigor Mortis

The main chemical changes in the muscle after death are the production of lactic acid by anaerobic glycolysis, the breakdown of creatine phosphate (CP) and finally the breakdown of adenosinetriphosphate (ATP) itself. Both glycolysis and the breakdown of CP are mechanisms for the re-synthesis of ATP from adenosinediphosphate (ADP), in the first case in the proportion of one-and-a-half molecules to every molecule of lactic acid formed, and in the second at the expense of one molecule of CP through the Lohmann reaction (Lohmann, 1935; Needham, 1942; Kuby, Noda & Lardy, 1954):

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow[\text{phosphokinase}]{\text{creatine}} \text{creatine} + \text{ATP}$$

When we find these reactions proceeding, but the ATP level remaining high and constant as in the early stages after death, it follows that ATP must be dephosphorylated in proportion to its re-synthesis. This balance between breakdown and re-synthesis, however, can be maintained only so long as the store of CP lasts. Beyond that point dephosphorylation increasingly exceeds re-phosphorylation so that the actual ATP level itself begins to fall, whether glycolytic re-synthesis is proceeding or not (Bendall, 1951; Lawrie, 1953). At this stage, too, ammonia and inosine monophosphate (IMP) appear in amounts equivalent to the amounts of ATP that disappear, so that it is probable that the following chain of reactions then occurs:

$$\text{ATP} \longrightarrow \text{ADP} + \text{P} \qquad \text{ATPase}$$

$$2\text{ADP} \underset{(\longleftarrow)}{\longrightarrow} \text{ATP} + \text{AMP}^* \qquad \text{myokinase}$$

$$\text{AMP} \longrightarrow \text{IMP} + \text{NH}_3 \qquad \text{deaminase}$$

* adenosinemonophosphate

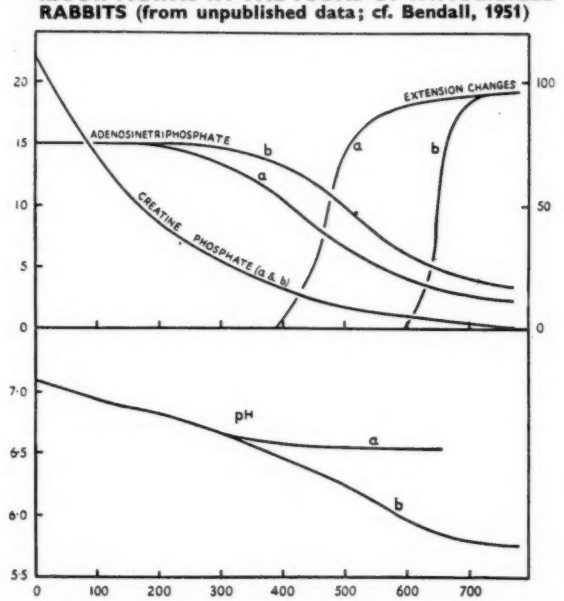
with the result that the adenine nucleotide supply quickly fails (Webster, 1953; Davey, unpublished).

We can regard the dephosphorylation of ATP as the primary reaction determining the rate of the other chemical changes. Its maximum rate in rabbit muscle during the onset of rigor at 17° C., however, is very low, being only of the order of 0.3 μatoms ~ P (labile P of ATP, ADP or CP) per min. per g., which represents less than $\frac{1}{100}$ of the potential ATPase activity of isolated myofibrils (Bendall, 1951; Perry, 1956; Webster, 1953). The enzyme responsible for this dephosphorylation is still not identified with certainty (Bate-Smith & Bendall, 1947, 1949; Marsh, 1952b; Webster, 1953), but, whatever its nature, its activity is markedly dependent on pH, being minimal at pH 6.7 and maximal at pH 7.2 and 6.0. No such maxima and minima are found with the ATPase of the fibrils, where the activity is minimal at pH 6.0 and maximal at about pH 7.4 (Bendall, unpublished data).

From what has been said, we see that the disappearance of ATP from the muscle, on which the physical changes depend, will be largely determined by the magnitude of the initial stores of CP and of glycogen, which together may be looked upon as the ATP potential. The level of this potential immediately before death will, in its turn, depend on the level of feeding or exercise, being high in well-fed and rested animals, lower in starved animals and almost zero in hunted or exhausted animals, whereas its level after death will depend on the precise manner of death, being drastically reduced when a violent struggle occurs. These various conditions can be simulated experimentally in animals such as the rabbit; this is particularly suitable on account of its size. Thus we can contrast the effect of a quiet death, for example by decapitation while immobilized with substances causing paralysis such as myanesin (Berger & Bradley, 1947), with the effect of more violent death, as by stunning and decapitation or after insulin convulsions. These are illustrated in figs. 2, 3.

In the case of a quiet death (fig. 2), we see that the initial values of CP and of pH are high and the changes are prolonged. Here, moderate depletion of the glycogen store by starvation (group a) has no detectable effect on the disappearance of CP which sets in from the moment of death, but it

FIG. 2. PHYSICAL AND CHEMICAL CHANGES DURING RIGOR MORTIS IN THE PSOAS OF IMMOBILIZED RABBITS (from unpublished data; cf. Bendall, 1951)



Temperature: 17° C.; nine muscles in each group

a: curves showing results in group of animals starved for 48 hr. before death

(Average initial glycogen ± S.E. = 3.2 ± 0.3 mg./g.; average final pH ± S.E. = 6.55 ± 0.03)

b: curves showing results in group of well-fed animals

(Average initial glycogen ± S.E. = 7.9 ± 0.3 mg./g.; average final pH ± S.E. = 5.75 ± 0.03)

Upper set of curves:

Abscissae: time after death (min.)

Left-hand ordinates: ATP and CP (μatoms ~ P/g.)

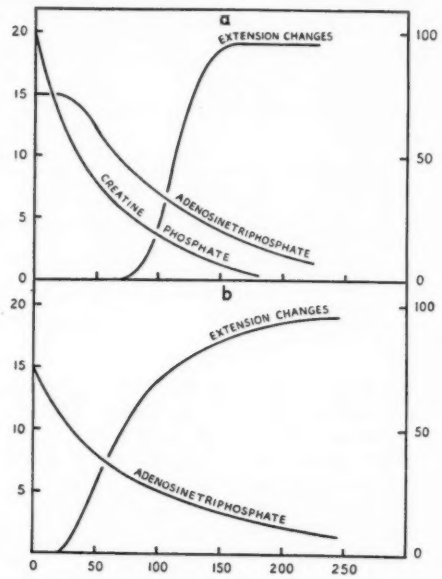
Right-hand ordinates: loss of extensibility as percentage of initial extensibility

Lower set of curves:

Abscissae: time after death (min.)

Ordinates: pH

FIG. 3. PHYSICAL AND CHEMICAL CHANGES DURING RIGOR MORTIS IN THE PSOAS OF RABBITS KILLED WITHOUT IMMOBILIZATION (from unpublished data; cf. Bendall, 1951)



Temperature: 17° C.

a: curves showing results in insulin-treated animal
(No initial glycogen; pH = 7.20 throughout)

b: curves showing results in three well-fed animals
(No initial creatine phosphate; range of initial glycogen = 2.5–3.4 mg./g.; range of initial pH = 6.38–6.46; range of final pH = 5.90–5.98)

Upper set of curves:
Abscissae: time after death (min.)
Left-hand ordinates: ATP and CP (μatoms ~ P/g.)
Right-hand ordinates: loss of extensibility as percentage of initial extensibility

Lower set of curves:
Abscissae: time after death (min.)
Left-hand ordinates: ATP (μatoms ~ P/g.)
Right-hand ordinates: loss of extensibility as percentage of initial extensibility

reduces the half-life of the ATP by about 100 minutes. In both groups, however, the ATP level remains high for 200 minutes after death, and is not completely exhausted even after 700 minutes. In contrast to these slow changes, we see that a violent death, either after insulin convulsions which deplete the glycogen store (fig. 3a) or by stunning and decapitation (fig. 3b), results in the very rapid disappearance of ATP, accompanied in the first case by loss of CP, but no glycolysis, and in the second by glycolysis alone. These effects are, of course, more pronounced in muscles such as the psoas, which actively participate in the struggle at death, than in others such as the longissimus dorsi or the pectoralis superficialis which are more quiescent (Bate-Smith & Bendall, 1949; Lawrie, 1953). This applies as well in the case of insulin-treated animals, that is, exhausted animals, as in the case of well-fed animals, killed while they were struggling. For instance, in one experiment the psoas of an exhausted animal contained no glycogen at death so that the pH remained high at 7.20, whereas the longissimus dorsi contained 0.2% of glycogen and the pH fell from 6.90 to 6.55 during the course of rigor (Bendall, unpublished results).

From these results it is clear that, in maintaining the ATP level, neither CP nor glycogen is alone effective, but only in combination, and that the greatest effect is obtained, per unit of ATP re-synthesized, when the glycogen store is just sufficient to last until the CP store is exhausted (see curves marked a, fig. 2).

The time-course of glycolysis itself is illustrated in the lower half of fig. 2 as the pH–time curve. The fall of pH has been preferred as a measure of glycolysis because it is more easily and accurately measured than either the lactic acid (LA) or the glycogen content (Bendall, 1951; Lawrie, 1953; Marsh, 1954). Within the limits of pH 7.30 to 5.70 it is linearly related to the formation of lactic acid, and hence to the re-synthesis of ATP, by the following equations, re-calculated from the line of best fit to the results quoted by Bate-Smith & Bendall in 1949:

$$\frac{\Delta LA}{\Delta pH} = 5.85 \text{ mg./pH/g. of muscle}$$
$$= 65 \text{ μequiv./pH/g. of muscle}$$
$$\equiv 97.5 \text{ μatom ATP—P/pH/g. of muscle}$$

The curve illustrates the complete course of glycolysis in the psoas muscles of well-fed and immobilized rabbits. Besides representing the case of such high glycogen stores, however, it also holds closely for the pH ranges traversed in the muscles of starved animals or animals killed without immobilization (Bate-Smith & Bendall, 1949). From this it follows that, knowing only the initial pH and the initial glycogen, we can predict the course of chemical change with fair accuracy, as Marsh (1954) has shown in the case of beef muscle.

Besides the phosphorylative pathway of glycolysis, discussed above, another mode of breakdown of glycogen in muscle has recently become of considerable practical importance. This takes place by the combined action of α-amylase, amylo-1:6-glucosidase and maltase to yield free glucose (Petrova, 1946, 1947; see Callow, 1955), and proceeds at about one-tenth the rate of the phosphorylation cycle. It becomes of importance, therefore, only when the stock of glycogen is so high that some of it is left over when the ultimate pH is reached, that is, the so-called " residual glycogen " (Lawrie, 1955). The contribution of this mode of breakdown to the post-mortem changes was entirely overshadowed by that of phosphorylative glycolysis, until the presence of free glucose in the muscles of various species post rigor was revealed by a search carried out by J. G. Sharp for the substances responsible for the Maillard browning reaction during the drying of meat (see Callow, 1955).

3. Relation of the Physical to the Chemical Changes

The extension changes, occurring in the rabbit psoas under the various treatments, are plotted as loss of extensibility against time in figs. 2, 3, from which it is at once clear that the relation of these changes to the chemical change is far from simple. The loss of ATP bears the closest relationship to the loss of extensibility. Taking the immobilized animals

first (fig. 2), we see that the extension changes in the group with depleted glycogen stores (a) do not set in simultaneously with the loss of ATP, but are delayed until the ATP level has fallen from 15 to 10 μatom ~ P/g., and are then completed as the level falls to 5 μatom ~ P/g. The extension changes are even more delayed in the group with high stores of glycogen (b), and set in only when the ATP level reaches 6 μatom ~ P/g., and are complete when the level has fallen to 4 μatom ~ P/g. Similar effects are found in the exhausted animals and the animals killed without immobilization (fig. 3). In fact, it seems a general rule, common to rabbit, horse, beef and whale muscle, that stiffening cannot occur until the ATP level has fallen more or less drastically (Bendall, 1951; Marsh, 1952a, 1954; Lawrie, 1953). At first sight this is surprising until it is remembered that even lower levels of ATP are sufficient to reverse rigor in fibre models, providing that the relaxing factor of Marsh (1952b) is also present (Bendall, 1952; Hasselbach & Weber, 1953). It is more surprising, however, first that the level at onset in the intact muscle varies according to the amount of glycolysis, and secondly that a very small further change in the level has so much subsequent effect on the extensibility. Although it is not possible to discuss the details here, these anomalies may be resolved by considering the turnover of ATP, rather than the level. Thus, the results of the work of Bendall (1951) on rabbit psoas and of Lawrie (1953) on the psoas, longissimus dorsi and diaphragm muscles of the horse have this in common, that the final fall in the rate of ATP turnover coincides very closely in every case with the onset of stiffening. This is illustrated in fig. 4. If this correlation is found to hold in other cases, then stiffening must depend not solely on a particular level of ATP, but on a lowered level associated with a decay of re-synthesis.

4. The Temperature Coefficient of the Chemical and Physical Changes

In the rabbit psoas, for which we have data relating to fall of pH, CP and ATP at two temperatures, it is possible to estimate an approximate $Q_{10°C.}$ for these changes as well as for the time of onset of stiffening. These coefficients have been calculated in Table I from the times for half-change of the CP, ATP and extension values. The data at 17° C. are taken from figs. 2 and 3, and those at 37° C. from unpublished data similar to those quoted by Bendall (1951). The average $Q_{10°C.}$ is close to the value of 1.58 given by Bate-Smith & Bendall (1949) from an independent set of data, and also to that obtained from pH–time curves.

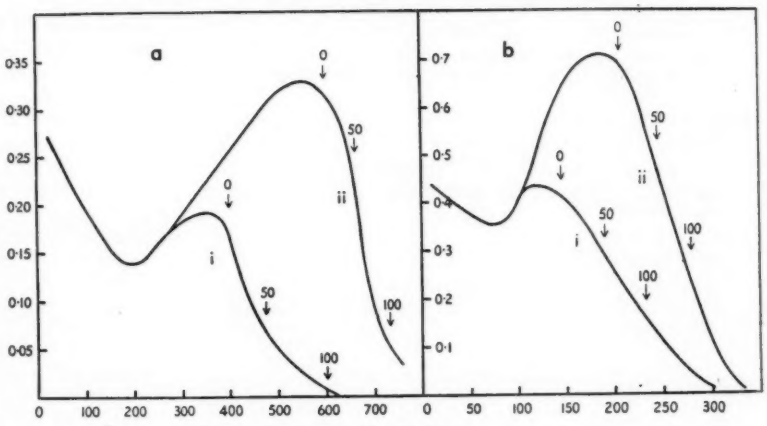
It is interesting to compare these coefficients for rabbit muscle with those given by Marsh (1954) for beef muscle, where the $Q_{10°C.}$ is itself highly dependent on the temperature, and rises particularly rapidly in the range 25–40° C., as seen in fig. 5. A similar dependence on temperature was noted over limited ranges in rabbit muscle by Bate-Smith & Bendall (1949). In beef, however, the $Q_{10°C.}$ over the range 17–37° C. is very high, being 1.90 compared with 1.55 in the rabbit. Since the absolute rates of chemical and physical change at 37° C. are about the same in the two species, it follows that, at 17° C., beef muscle will take nearly one-and-a-half times as long as rabbit muscle to pass into rigor under the same conditions.

The changes in whale muscle are even slower than in beef, and in some cases differ not only in rate but also in kind. For

TABLE I. CALCULATION OF THE $Q_{10°C.}$ FOR THE PHYSICAL AND CHEMICAL CHANGES AFTER DEATH IN THE PSOAS OF IMMOBILIZED RABBITS
(see text, section 4)

Parameter	Time for half-change (min.)		$Q_{10°C.}$
	17° C.	37° C.	
Creatine phosphate level	150 150	63 63	1.55
Adenosinetriphosphate level	470 570	198 234	1.55 1.56
Extensibility of muscle	474 654	201 260	1.55 1.56
		Average	1.55

FIG. 4. ONSET OF STIFFENING IN RELATION TO RATE OF TURNOVER OF ATP IN RABBIT AND IN HORSE MUSCLE



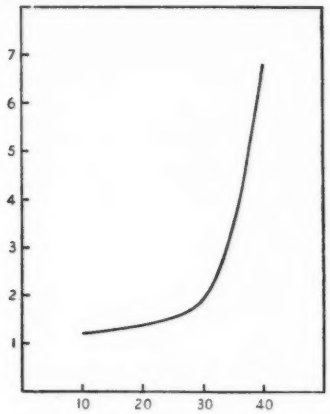
Abscissae: time after death (min.)
Ordinates: ATP turnover (μatoms ~ P/min./g.)

Rate of turnover of ATP is calculated from the rates of re-synthesis of ATP from glycolysis and CP to which is added the rate of net loss of ATP.
Arrows indicate 0%, 50%, and 100% loss of extensibility.

a: rabbit psoas at 17° C.
curve i: final pH 6.55
curve ii: final pH 5.75
(Rates calculated from fig. 2.)

b: horse muscle at 37° C.
curve i: diaphragm; final pH 5.76
curve ii: longissimus dorsi; final pH 5.51
(Rates calculated from published values of Lawrie, 1953.)

FIG. 5. TEMPERATURE COEFFICIENT ($Q_{10°C.}$) OF THE pH CHANGES AFTER DEATH IN BEEF MUSCLE, PLOTTED AGAINST TEMPERATURE (values taken from Marsh, 1954)



Abscissae: temperature (°C.)
Ordinates: $Q_{10°C.}$

instance, Marsh (1952a) records a case where the pH, ATP level and extensibility of a whale psoas stayed high and constant for as long as 18½ hours at 22° C., and then abruptly began to decrease at about the rate characteristic of rabbit muscle. Webster (personal communication) has encountered similar instances. At present there is no satisfactory explanation of the phenomenon. It would, however, seem to be of great general interest in the biochemistry of muscle.

5. Other Physical and Chemical Changes Associated with Stiffening

Many other chemical and physical changes doubtless occur concomitantly with those reported here, but space permits only the inclusion of two which seem to be of outstanding importance: the changes in electrical resistance and the changes in redox potential. The resistance changes of muscle as it passes into rigor have been studied by Callow (1938) and Rowan (1940). It appears from their work that the resistance of the muscles of well-fed animals falls as stiffening sets in, the main part of the fall being associated with the low pH at that time. The fall is not necessarily associated with stiffening as such, however, because it does not occur in the muscles of insulin-treated animals (Rowan, 1940) or in iodoacetate-poisoned muscles (Bozler & Cole, 1935), where the pH remains high throughout the course of rigor. For these reasons, the fall appears to be due solely to the effect of a low pH on the water relations of the fibre, and not, for instance, to loss of ATP from the muscle, with which it clearly cannot in general be correlated. On the basis of this change, and of the greater permeability of the muscle to salt when rigor is complete, Callow refers to the " open structure " of post-rigor muscle at low pH values ($\sim$ 5.60) compared with the " closed structure " characteristic of higher pH values ($>$5.80). In the case of " open structure ", the muscle actually loses fluid, indicating that considerable damage has occurred to the membranes.

Another change which occurs after death is the fall in the redox potential, E_h, which has been studied by Barnes & Ingram (1955) in relation to the growth of bacteria in muscle. They showed very clearly that the main fall in E_h from positive to negative values occurs during the first stages of rigor while the pH is falling from 7.0 to 6.6, that is, during the phase of CP disappearance (cf. fig. 2). If the muscle is kept sterile by means of aureomycin, very little further change occurs, but when the bacteria normally present in the animal are allowed to multiply, there is first a plateau in the E_h–time curve and then a further considerable fall, which is evidently due to the growth of facultative anaerobes and clostridia, but which finally favours the growth of clostridia only. The theoretical interpretation of these E_h changes, particularly the first fall, is at present very difficult, because there is insufficient evidence of their relation to the other post-mortem changes, but it is probable that the suggestion of Barnes and Ingram is correct—that they are due to the consumption of the last traces of oxygen by the muscle.

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Book Reviews

Neuro-Vascular Hila of Limb Muscles

James Couper Brash. Edinburgh: E. & S. Livingstone Ltd., 1955. xvi + 100 pages; 30 plates. 26 × 19 cm. £1 10s.

The important data gathered together in this extremely useful book have been collected over a period of many years, and the results contained in the book have been known to many since 1945, when a limited number of copies of the data for the upper limb were circulated. It is a pleasure to have now the full data for both upper and lower limb (hand and foot are not included) in one clear and concise volume.

The introduction to the atlas includes a short review of the previously published observations on the site of nerve entry into limb muscles. These earlier observations were concerned almost entirely with the point of nerve entry and were to a large extent extensions and clarifications of the original work by Duchenne in 1867 in determining the " motor points " of the more superficial muscles. As the primary object of the text under review, to quote the preface, " was to determine the extent to which the principal blood-supply of the muscles is associated with the nerves, *i.e.*, the constancy or otherwise of a definite ' neuro-vascular hilum ' ", this is a valuable extension of the older work which, as we have said above, is concerned more directly with simple motor innervation.

From the large amount of material which Professor Brash has collected from the Departments of Anatomy of Aberdeen, Leeds, Glasgow and Edinburgh, the actual observations are drawn together in a most attractive and practical form. Each motor nerve is taken in succession, and the individual muscle or group of muscles supplied by this nerve is illustrated in clear semi-diagrammatic form. By the side of each diagram is a scale of reference in relationship to some prominent and obvious bony joint. The mode of entry of the nerve and the vessels in the modal distribution is illustrated in these diagrams. On the page opposite to each diagram, in addition to short explanatory notes, the position of the main neurovascular entry is given with its percentage occurrence and the limits of variation of this position. For comparison with these findings, the author summarizes the findings by earlier writers on the nerve distribution to the same muscle.

There can be no doubt that these carefully and clearly set-out data have been—and will continue to be—of greatest importance to anyone concerned with the surgery of the limbs, and for such purposes the mode of presentation forms a most admirable reference. Variation, as would be expected, is common, but the limits of variation are surprisingly small. It is interesting to note that the two muscles that have the most constant positioning of the neurovascular hila are biceps brachii and the gastrocnemius muscles, in both of which the modal position accounts for more than 90% of the specimens examined.

In this atlas Professor Brash makes no attempt to deduce laws or to make sweeping generalizations to cover the distribution of neurovascular hila, such as were attempted in the late 19th century. This adds advantage to what is a thoroughly practical book for anatomists and physiologists, physiotherapists and surgeons, who will remain indebted to Professor Brash for this beautifully produced piece of work.

G. Causey

An Atlas of Musculoskeletal Exposures

H. F. Moseley. Philadelphia & Montreal: J. B. Lippincott Company, 1955. xii + 235 pages; 71 plates + 63 figures. 29 × 22 cm. £9

This atlas is concerned with the surgical approach to bones and joints. The first part of the text consists of a short synopsis of the general principles underlying such an approach, including the preparation of the operative area and a very general consideration of skin incisions and the " no-touch " technique. The approaches to the face, the upper extremity, the trunk and the lower extremity are then dealt with systematically, and each of the incisions that are in current use are illustrated by generous coloured plates. In a systematic discussion of such incisions it is inevitable that there will be difficulty in giving greater emphasis to the more commonly adopted procedures than to those which will be used only on rare occasions.

The section on each region begins with a short statement of the main conditions for which operative intervention is likely to be required and is followed by careful notes on skin preparation and posturing. Each incision is then illustrated to show the anatomical structures that will be met with in the exposure. These illustrations have been accumulated over a long period from dissected material, post-mortem material, and from actual operations, and the beautifully coloured illustrations by their clarity and completeness do great credit to both the author and the artist. In the opinion of the reviewer, it is these visual demonstrations of the anatomy of the approach which will recommend this book to all who are concerned with the surgery of bones and joints.

There are short lists of references at the end of each section, and a useful addition to this list is a reference to the available films on the subject (the availability applies, of course, to the United States of America).

With regard to particular sections, the one concerned with the trunk approaches is particularly useful and stimulating, whilst that on the hand contains an interesting diagram of correct and incorrect incision; but it seems a pity that in the first sentence of this section the tactile function of this organ is not mentioned.

One might criticize the repetition of details about skin preparation, draping and posturing, but by taking these subjects in relationship to each region the various sections of the book become self-contained and its value therefore as a book of reference is considerably increased. To the anatomist again the illustrations will be of extreme value and use, particularly in that many of the dissections illustrated are taken from angles which are perhaps not as familiar as they should be to those dealing with the cadaver. This beautifully produced book will be an essential part of the library of both surgeons and anatomists.

G. Causey

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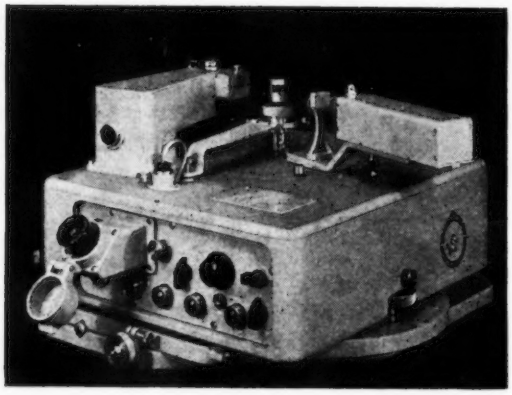

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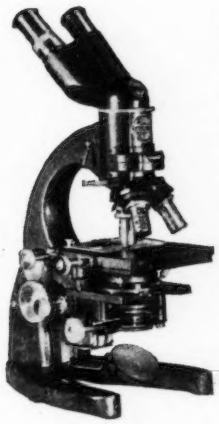

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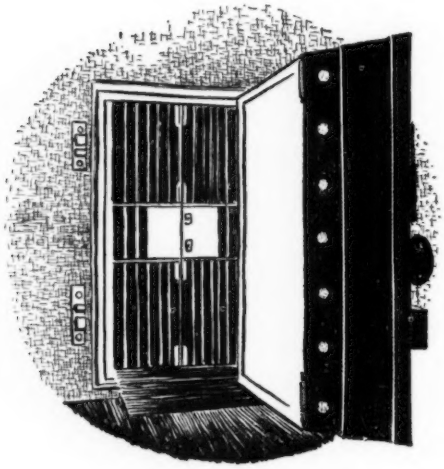

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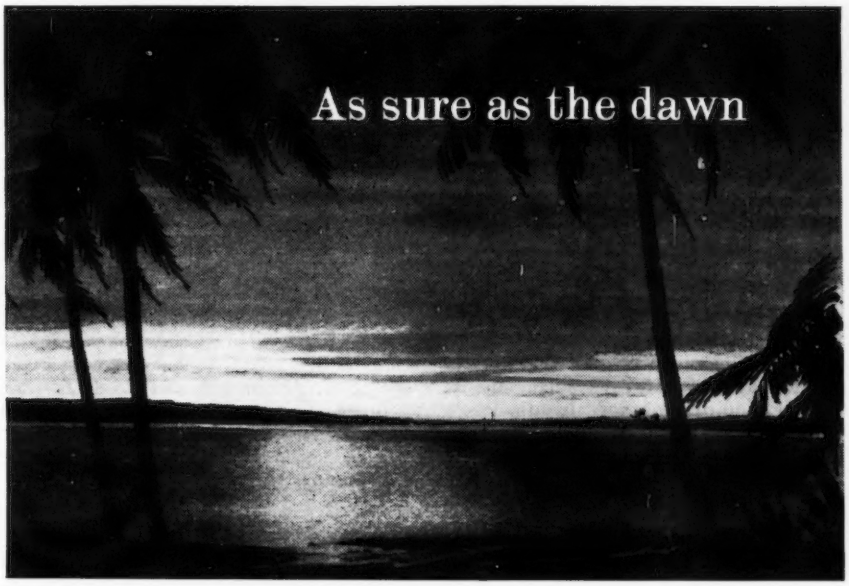

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